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Vertebrate retinal ganglion cells are selected from competent progenitors by the action of *Notch*

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SUMMARY

The first cells generated during development of the vertebrate retina are the ganglion cells, the projection neurons of the retina. Although they are one of the most intensively studied cell types within the central nervous system, little is known of the mechanisms that determine ganglion cell fate. We demonstrate that ganglion cells are selected from a large group of competent progenitors that comprise the majority of the early embryonic retina and that differentiation within this group is regulated by *Notch*. *Notch* activity in vivo was diminished using antisense oligonucleotides or augmented using a retrovirally transduced constitutively active allele of *Notch*. The number of ganglion cells produced was inversely related to the level of *Notch* activity. In addition, the Notch ligand Delta inhibited retinal progenitors from differentiating as ganglion cells to the same degree as did activated Notch in an in vitro assay. These results suggest a conserved strategy for neurogenesis in the retina and describe a versatile in vitro and in vivo system with which to examine the action of the Notch pathway in a specific cell fate decision in a vertebrate.

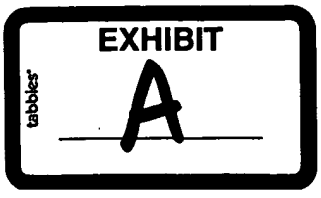
Key words: retina, ganglion cells, *Notch*, equivalence group, vertebrate

INTRODUCTION

At the earliest stages of development, the anlage of the vertebrate central nervous system (CNS) is composed entirely of mitotic progenitors. Little is known about the mechanisms by which these cells become determined to their fates and transform into the myriad differentiated cell types characteristic of the mature CNS. The retina is an attractive region of the CNS in which to study these issues because it is a relatively simple structure with well-characterized cellular architecture and is accessible to experimental manipulation (Dowling, 1987). Retinal lineage analysis has shown that progenitors are capable of producing overlapping combinations of cell types, suggesting that progenitors may be multipotential and that signals in a progenitor's environment contribute to the determination of cell fate (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990; Fekete et al., 1994). A number of diffusible factors have been shown to be capable of affecting differentiation of late-born cell types in the retina, including taurine (Altshuler et al., 1993) and 9-*cis* retinoic acid (Kelley et al., 1994), though their roles in normal development remain obscure.

The first cells produced during retinal development are the ganglion cells, the projection neurons of the retina. Extensive study of ganglion cell physiology (Kuffler, 1953; Dowling, 1987) and projections (Sperry, 1963; Bonhoeffer and Gierer, 1984) have demonstrated that ganglion cells transduce the retina's analysis of visual information and topographically map onto secondary visual centers in the brain. Despite this wealth of functional data, little is known about how ganglion cells become committed to their fates during development. Ganglion cells have a number of features that make them attractive as a model for fate acquisition in the CNS. Because ganglion cells, like other CNS projection neurons, are born first during development, they arise from an environment that lacks other differentiated cells, thus simplifying cell type identification and possibly the complexity of environmental influences on cell fate. Ganglion cells also share antigenic, morphologic and functional characteristics with other CNS projection neurons and multiple markers exist with which to identify them in vivo and in vitro (Dowling, 1987).

In several invertebrate species, analogous early-born neurons are generated from a pool of progenitors with equivalent developmental potential, termed an 'equivalence group' (Kimble et al., 1979). Cell-cell interactions inhibit most of the cells in an equivalence group from differentiating into a primary cell fate and most take on secondary or tertiary fates. Only the cells that escape inhibition differentiate into the primary cell type. In *Drosophila*, proneural basic helix-loop-helix genes of the *achaete-scute* complex *(as-c)* (Skeath and Carroll, 1991, 1992) or *atonal* (Jarman et al., 1994) are required for establishment of neural competence of cells in an equivalence group. The inhibition of differentiation results from down-regulation of the *as-c* loci by the action of the neurogenic gene *Notch (N)* (Cabrera, 1990) and multiple other genes implicated in the transmission of the *N* signal, including *mastermind*, *deltex*, *Hairless*, *Suppressor of Hairless* and the *Enhancer of Split* complex (reviewed by Artavanis-Tsakonas et al., 1995). Definition of equivalence



groups has been accomplished by eliminating cell-cell interactions that normally mediate inhibition of differentiation by either generating neurogenic loss-of-function mutants (Heitzler and Simpson, 1991; Goriely et al., 1991) or ablating one or more cells in the equivalence group (Kimble, 1981). Elucidation of progenitor competence and the mechanisms of cellular specification in the vertebrate CNS have been more difficult, though evidence exists for a two-cell equivalence group in the zebrafish spinal cord (Eisen, 1992).

In the retina of *Drosophila*, genetic mosaic analyses demonstrated that, similar to the vertebrate retina, a progenitor's lineage did not predict the cell types that it produced (Ready et al., 1976; Lawrence and Green, 1979). Multiple cell fate decisions in the *Drosophila* retina have been shown to depend upon interactions among neighboring cells (Cagan, 1993). The R8 photoreceptor is the first cell type to terminally differentiate in the *Drosophila* retina, and the generation of the correct number and spacing of R8 cells requires *Notch* and other genes of the neurogenic group (Cagan and Ready, 1989; Baker et al., 1990; Baker and Rubin, 1992). Reduction of *Notch* activity in the region of the eye imaginal disc in which R8 differentiation is taking place promotes the immediate differentiation of most of the cells in the region as neurons with characteristics of R8, leading to the suggestion that these cells represent a large R8 equivalence group (Cagan, 1993).

In this paper, we show that the first-born neurons in the chick retina, the ganglion cells, are generated by a similar strategy to that operative in development of R8 in the *Drosophila* retina. The function of a critical component of the regulation of this developmental decision, Notch, is also conserved. The majority of progenitors in the early retina were found to be competent to differentiate as ganglion cells. Chick *Notch-1* was found to be expressed in the undifferentiated cells of the retina at this stage and alteration of *Notch* activity in either direction affected the number of ganglion cells produced in the retina. Antisense oligonucleotides were used to decrease Notch-1 level in vivo and in vitro, increasing recruitment into the ganglion cell pathway and doubling the thickness of the ganglion layer in vivo without affecting mitotic activity. Conversely, retroviral transduction of constitutively active Notch into progenitors decreased recruitment into the ganglion cell pathway in vivo and in vitro, and this effect could be eliminated by antisense treatment. Finally, *Drosophila* Delta was found to substitute for a putative Notch ligand in blocking differentiation of chick retinal progenitors into ganglion cells in a co-culture assay. The experimental system that we describe makes possible the manipulation of the Notch pathway activity in vivo and in vitro, with quantifiable effects on the differentiation of a specific vertebrate cell type.

MATERIALS AND METHODS

Experimental animals

White Leghorn chicken eggs were purchased from Spafas, Inc. (Norwich, CT) and incubated at 38°C in a rotating humidified incubator. Staging was done according to Hamburger and Hamilton (1951).

Retinal cell culture

The protocol for dissociation of retinal cells and culture in collagen gels was modified from Altshuler and Cepko (1992); cells were cultured at a concentration of 25,000 cells/25 μl gel unless otherwise noted, with the addition of 100 μg/ml conalbumin (Sigma) and 10 μg/ml insulin. Gels were dissolved by addition of media containing 200 units/ml collagenase (Worthington); cells were transferred to polyornithine-coated slides and fixed in 4% paraformaldehyde in PBS for 15 minutes.

Cell pellets were made by centrifuging 10^6 cells at 8000 *g* for 10 minutes. For explant cultures, retinae were dissected and transferred directly to 200 μl medium. For marking of S-phase cells, 5 μCi/ml [^{3}H]thymidine (Amersham) was included in the medium of explants for 1-2 hours before dissociation.

Immunohistochemistry and cell counting

After fixation, cells were blocked for 1 hour in 10% FCS, 5% donkey serum (Jackson Immunologicals, West Grove, PA) and 0.4% Triton X-100 in PBS. Primary antibody incubations were for 1 hour at room temperature in 10% donkey serum in PBS. Monoclonal antibodies and dilutions used to detect ganglion cells were as follows: 8A1, recognizing low molecular weight neurofilament (Barnstable, 1987), 1:1000; RA4 (McLoon and Barnes, 1989), 1:300; 4D5, recognizing the Islet-1 protein (Yamada et al., 1993), 1:50; 40.2D6 (directed against Islet-1) and 3A10 and E/C8 (both directed against neurofilament-associated proteins), obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, 1:10; RMO 270.7, directed against low-molecular weight neurofilament (Carden et al., 1987), 1:10; 8D9, directed against NgCAM (Lemmon and McLoon, 1986), 1:10; anti-GAP-43 (Sigma Immunologicals), 1:1000. Polyclonal rabbit sera used were: anti-α_6-cytoA and anti-α_6-cytoB, directed against α_6 integrin (de Curtis and Reichardt, 1993), 1:500 and anti-Trk B (Santa Cruz Biotechnology, Santa Cruz, CA), 1:50. Each antibody was used to stain chick retinal sections from stage 14 (E1.5) to stage 35 (E8) and were found to be ganglion cell-specific. Rabbit anti-p27 gag antiserum (Spafas) was used at 1:100. Secondary antibodies used were Texas Red-conjugated donkey anti-mouse IgG, fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson Immunologicals), or anti-mouse Vectastain ABC Elite (Vector Labs), using DAB as chromogen. For autoradiography, slides were processed for immunocytochemistry, then dehydrated through graded alcohols, dipped in autoradiography emulsion (Kodak NTB-2) and exposed in light-tight boxes at 4°C for 2 days. After staining, slides were mounted in Gelvatol and coverslipped, and observed under UV illumination on a Zeiss axiophot microscope. At least 200 cells were counted in each well of a slide; each experimental condition was performed and counted in triplicate in each experiment, and each experiment was repeated at least three times.

Retinal cell:S2 cell co-culture

Drosophila S2 cells and L49-3-6 Delta transformants were a gift of Dr Spyros Artavanis-Tsakonas, Yale University, New Haven, CT and were cultured according to Fehon et al. (1990). *Drosophila* and chick cells were mixed at a ratio of 50:1 (*Drosophila*:chick), spun at 8000 *g* for 10 minutes and the pellets cultured in 200 μl retinal media in a humidified 5% CO_2 atmosphere at 37°C for 24 hours. After incubation, pellets were dissociated, fixed, stained for NF and processed for autoradiography. Counts of the percentage of [^{3}H]thymidine-labelled cells that also expressed NF were made. 3 pellets using untransfected S2 cells and 3 pellets using S2-Delta cells were made in each of 3 experiments; 100 cells were counted in triplicate for each experiment.

In situ hybridization

The in situ hybridization procedure was modified from Riddle et al. (1993), as follows: retinae were sectioned at 15 μm and permeabilized with proteinase K (Boehringer-Mannheim, 1 μg/ml) for 10 minutes. The probe was a digoxigenin-labelled 1 kb chick *Notch-1* mRNA encoding a portion of the extracellular domain between the

EGF repeats and *lin12/Notch* repeats, transcribed from a plasmid supplied by Domingos Henrique and Julian Lewis at the Imperial Cancer Research Fund, Oxford, UK.

Western blotting

Retinal extracts were made by resuspending stage 24 chick retinae in RIPA buffer containing 3.4 μg/ml aprotinin, 25 μM leupeptin, 1 μg/ml pepstatin and 100 μM PMSF. 100 μg of total protein were run per lane, as determined by a bicinchoninic acid microassay kit (Pierce). Filters were incubated overnight at 4°C with an affinity-purified rabbit polyclonal antiserum directed against synthetic peptides from the cdc10/ankyrin repeat region of TAN-1 (a gift of J. Aster and J. Sklar) diluted 1:100. Specificity of the antiserum was assessed by preincubating the antibody with 1 mg/ml of the peptides against which the antibody was made, overnight at 4°C. Secondary antibody was 0.6 μCi/ml ^{125}I-labelled sheep anti-rabbit (Amersham) and signal was visualized using a PhosphorImager (Molecular Dynamics).

Antisense oligonuclotides

The sequences of *Drosophila Notch*, *Xotch*, rat *Notch 1* and *2*, *Motch* and *TAN-1* were obtained from GenBank. Unpublished sequence data on *TAN-1* was obtained from Drs Jon Aster and Jeffrey Sklar at the Brigham and Women's Hospital, Boston. Unpublished partial sequence data on the epidermal growth factor-like repeat region and the *lin-12/Notch* repeat region (see Fig. 6A for explanation of terms) of a chicken *Notch-1* homologue (*CNotch-1*) was very kindly provided by Domingos Henrique and Julian Lewis, Imperial Cancer Research Fund, Oxford, UK. Antisense oligonucleotides were 23-mers with phosphothiorate linkages between all bases, were approximately 50% GC and had no base repeated any more than twice in succession. Oligonucleotides were designed against three distinct regions of the *Notch* sequence: (a) the EGF repeat region (oligo designated EGFR). *CNotch-1* sequence was used, with antisense sequence 5′-GTAGTCATTGACCCGCTGCACGC-3′. (b) the lin12/Notch repeat region (oligo designated LNR), with *CNotch-1* antisense sequence 5′-CCAGCACTGCAGTGACTGTGAGC-3′. (c) the 5′ end of the cdc10/ankyrin repeat region (oligo designated CDCR). Chicken sequence was not available, so rat *Notch-1* sequence was used. Nucleotide sequence is highly conserved in this region and rat *Notch-1* sequence was used because it was most homologous to the chick *Notch-1* sequence in other regions. The CDCR oligonucleotide sequence differed at only 1-2/23 positions among the *Xenopus*, mouse, rat and human *Notch* homologues. The antisense sequence used was 5′-CCTCCGCTGCAGGAGGCAATCAT-3′, representing bp 5871-5893 of rat *Notch-1*. For the oligonucleotide mismatch experiments, the chick LNR region sequence was used. Nucleotide substitutions were made only in position one of a codon, as the chicken flock used is outbred and position one is least likely to vary among strains of a species. Reading frame of the chick sequence was determined by comparison with other *Notch* homologue sequences in which the start site and reading frame have been defined. A substitution in the antisense nucleotide sequence was then made in position 1 of one, three or five codons, with maintenance of GC content.

Syntheses were done at the Howard Hughes Medical Institute Biopolymer Facility, after which the oligos were purified by running over a NAP-10 column, desiccated in a spin-vac overnight and resuspended in a minimum volume of distilled water. OD_{260} readings were done to determine concentration. For each antisense oligonucleotide, a corresponding sense oligonucleotide was made and used in parallel in each experiment.

Injections of 5 μl of 250 μM oligonucleotide in DME were done into the vitreous and subretinal spaces of stage 16 embryos (E2; Hamburger and Hamilton, 1951), after Fekete and Cepko (1993), with harvest at stage 27 (E5). Neurogenesis is active thoughout the retina at these early stages, with central retina developing before peripheral. More ganglion cells would be expected in central regions and numbers of ganglion cells were therefore assessed independently in each region. In order to standardize the region of the eye analyzed, sections in which the diameter of the lens was the same were used and, on these sections, a central, intermediate and peripheral region, each with a specific retinal thickness, were counted. Because section thickness and magnification were also constant, this technique allowed the number of ganglion cells to be compared directly. Ganglion cells were identified on sections using antibodies to the Islet-1 protein, as this antigen was found to be completely specific to ganglion cells in the retina through E8 (C. P. A. and C. L. C., unpublished) and gives discrete nuclear staining which allows quantitation. Counting of individual cells in sections using NF or RA4 is not possible because both are process-associated and individual cells could not be distinguished. Counts were done of all Islet-1$^+$ cells in a high power field using a hemocytometer mounted on the microscope; 3 fields of each type were counted for each retina and 5 sense- and 5 antisense-injected retinae of each oligonucleotide type were analyzed.

Explants were incubated for 24 hours in 200 μl medium containing oligonucleotide at 37°C. Concentrations of oligonucleotide in medium from 0.1-100 μM, with or without 0.1-25 μM DOTAP, were tested initially to determine optimal concentrations. DOTAP was found to have no effect in any condition, so was omitted from later experiments. 3 sense and 3 antisense explants were used for each experiment and each experiment was repeated 3 times for each oligonucleotide type.

RNase protection assay

After 24 hours of explant culture in oligonucleotide, retinal RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction, according to established protocols (Chomczynski and Sacchi, 1987). RNase protections were done using the RPA II kit (Ambion, Austin, TX), according to the supplied protocol. 1-20 μg RNA was used for each reaction initially, with 5 μg being used for most experiments. The *Notch* probe was a 257 bp transcript from a *Pst*I digest of the chick *Notch-1* plasmid, with an expected protected region of 178 bp. The control probe was a 358 bp transcript from a *Pvu*II digest of a chick *EF1α* cDNA with an expected protected region of 116 bp, provided by Randy Johnson and Cliff Tabin, Harvard Medical School. Probes were labelled with α^{32}P-CTP and gel-purified. The RPA II kit protocol was followed, with the following modifications. Hybridizations were done at 45°C overnight and RNase digestions were done with 5 U/ml RNase A and 200 U/ml RNase T_1 at 37°C for 30 minutes. Protected fragments were separated on a 6% acrylamide/8M urea gel and quantitated using a PhosphorImager (Molecular Dynamics, Mountain View, CA).

Retroviral construction and injections

The cDNA JK5T corresponding to BP 4045-7948 of *TAN-1* (Ellisen et al., 1991), was a gift from Drs Jon Aster and Jeffrey Sklar at the Brigham and Women's Hospital, Boston. To make the TANIC vector, JK5T was digested with *Bss*hII and *Bsu*36I (Boehringer), and the resulting 2362 bp *TAN-1* fragment was blunted with T4 DNA polymerase and ligated into the *Cla*I site of RCAS(BP)A. pTANIC was transfected into line 0 chicken embryo fibroblasts by $CaPO_4$ precipitation. A control RCAS(BP)A-derived vector, APA, carrying the human placental alkaline phosphatase gene (Fekete and Cepko, 1993) was produced in parallel. Supernatants were harvested, concentrated by centrifugation at 20,000 *g* for 2 hours and frozen at −80°C until use. Viral titers were determined by infection of QT6 fibroblasts followed by staining for viral gag protein using monoclonal antibody 3C2 and HRP-conjugated donkey anti-mouse secondary antibody (Stoker and Bissell, 1987). Titer of TANIC was 10^8 colony forming units (cfu)/ml and the titer of the APA control vector was 10^7 cfu/ml.

Injections of TANIC and APA were done into stage 16 embryos (E2; Hamburger and Hamilton, 1951), as above for oligonucleotide

injections, except that 0.2-0.5 μl of virus suspension was injected into the subretinal space only, of the right eye. Quantitation of ganglion cells in retinal sections was done in the same way as for oligonucleotide-injected eyes. Infected regions of retina were identified by staining with antibodies to the p27 gag protein (Spafas). Counts were done of all Islet-1$^+$ cells in a high power field, in both infected and uninfected regions; 3 fields of each type were counted for each retina, and 5 TANIC- and 5 APA-infected retinae were analyzed.

RESULTS

The majority of retinal progenitors are competent to differentiate as ganglion cells

Neurogenesis in the chick retina occurs between E2 and E10; ganglion cells are born first, between E2 and E6 (Prada et al., 1991). Though less than 5% of retinal cells are ganglion cells in the adult chicken (Coulombre, 1955), the percentage of developing retinal cells that are ganglion cells was not known. This was determined by dissociating and immediately fixing chick retinal cells, followed by immunocytochemistry for the low molecular weight neurofilament (8A1; Barnstable, 1987), and the monoclonal antibody RA4 (McLoon and Barnes, 1989). Both antibodies recognize ganglion cells shortly after their terminal division in the retinal proliferative zone and remain specific for this cell type throughout retinal histogenesis (Barnstable, 1987; McLoon and Barnes, 1989; C. P. A. and C. L. C., unpublished observations). At E4 (stage 24), the

Fig. 2. Morphology of retinal cells in dissociated culture. Chick retinae at E4 (stage 24) were dissociated and cultured at low density for 24 hours in collagen gels, then stained in situ with the ganglion cell-specific marker RA4 (McLoon and Barnes, 1989). Only some cells and processes are in focus because of the three-dimensional nature of the culture system.

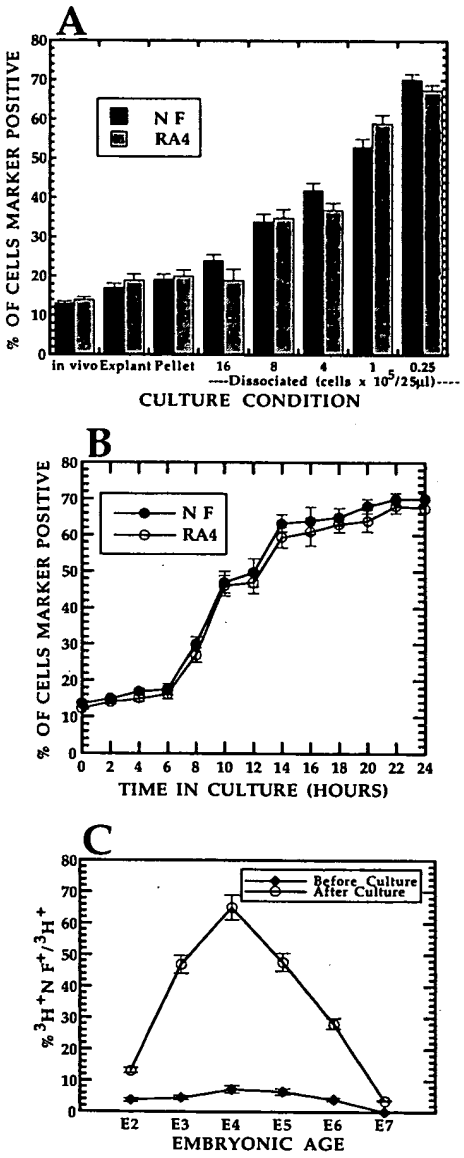


Fig. 1. (A) Density dependence of ganglion cell differentiation in culture. E4 (Stage 24) chick retinal cells were cultured for 24 hours under various conditions and stained for the ganglion cell-specific markers NF and RA4. The percentage of DAPI-stained cells that were NF$^+$ or RA4$^+$ were scored. Values plotted are means±s.e.m. of ≥3 experiments, with each condition done in triplicate in each experiment. 'in vivo', retinae left in the animal for 24 hours; 'explant', retinae cultured intact; 'pellet', retinae dissociated and reaggregated into a pellet of 10^6 cells, 'dissociated', retinae dissociated and placed in collagen gels at the indicated density. (B) Time course of ganglion cell marker expression in dissociated cultures. E4 (stage 24) chick retinae were dissociated and cultured at a density of 0.25×10^5/25 μl in collagen gels. Cells were harvested every 2 hours for the 24 hour culture period and stained for two ganglion cell-specific antigens, NF and RA4. (C) Developmental series of NF expression in dissociated cultures. Chick retinae from E2 (stage 16), E3 (stage 20), E4 (stage 24), E5 (stage 27) E6 (stage 29) and E7 (stage 31) were incubated as explants for 1 hour in 5 μCi/ml [^{3}H]thymidine, dissociated and cultured at a density of 0.25×10^5/25 μl in collagen gels for 24 hours, stained for NF and developed for autoradiography. $^3H^+$ cells that were also NF$^+$ were scored. 'Before culture', cells fixed and stained immediately after dissociation. 'After culture', cells cultured for 24 hours, then fixed and stained. The percentage of ^{3}H-labelled cells that were NF$^+$ is shown (mean±s.e.m. for 3 experiments).

peak of ganglion cell genesis, 13.6±0.5% (mean ± s.e.m.) were neurofilament $(NF)^+$ and 12.3±0.4% were $RA4^+$.

If cell-cell interactions were required for some aspect of ganglion cell differentiation, then the percentage of cells differentiating as ganglion cells might change with culture conditions that varied cell-cell contact. E4 retinae were cultured for 24 hours in serum-free conditions in 3 ways: as intact retinae (explants), as pellets (cells reaggregated through centrifugation), or as dissociated cells suspended in collagen gels at varied initial densities. The results of these experiments are in Fig. 1. From E4 (stage 24) to E5 (stage 27), the total number of cells in retinae left in vivo increased by 10-fold, but the percentage of cells that express NF or RA4 did not change (13.0±0.6% NF^+ and 14.0±0.7% $RA4^+$). Cells in explant culture, or cells dissociated and reaggregated into a pellet, showed a small increase, to 17-19% NF^+ or $RA4^+$. Cells cultured in collagen gels at the highest density showed a similar small increase in NF^+ or $RA4^+$ cells. As the cell density in collagen gels was progressively decreased over a 50-fold range, however, the percentage of cells that expressed NF^+ or $RA4^+$ after 24 hours increased (Fig. 1A). At the lowest density (0.25×10^5 cells/25 μl), most cells were separated from their neighbors by several cell diameters; in this condition, the number of NF^+ and $RA4^+$ cells increased 5-fold during the 24 hours in culture, to 70.2±1.4% NF^+ and 67.4±1.4% $RA4^+$.

At E4, over 90% of the cells in the chick retina are mitotic progenitors (Dutting et al., 1983) and thus most of the NF^+ and $RA4^+$ cells present after 24 hours must have arisen from cells that were mitotic at the beginning of the culture period. This was confirmed by including [^{3}H]thymidine in the culture medium to label mitotic cells. When only [^{3}H]thymidine-labelled cells were scored after 24 hours in the lowest density culture, 75.4±1.0% were found to be NF^+, similar to the result from counting all cells. To exclude the possibility that differential cell division was responsible for the observed increase in the number of NF^+ or $RA4^+$ cells during the culture period, cells in various culture conditions were labelled with [^{3}H]thymidine cumulatively throughout the 24 hour culture period. The percentage of cells that were labelled with [^{3}H]thymidine after culture at lowest density, highest density, pellet and explant were the same, showing that differential mitosis in low density cultures does not explain the observed increase in cells expressing ganglion cell markers. Similarly, to exclude the possibility that differential death of cells not bearing the ganglion cell markers was responsible for the observed increase, cell recovery was quantified for each culture condition. The number of cells recovered after 24 hours was 80-90% of the number recovered if the cultures were harvested immediately after being made and this percentage did not vary according to cell density in culture. Furthermore, greater than 90% of the cells recovered after 24 hours of culture were viable as assayed by trypan blue exclusion.

Given the large increase in cells expressing ganglion cell markers over the short time in vitro, the time course of marker expression in the lowest density culture condition was examined by harvesting cells every 2 hours, and performing NF and RA4 immunocytochemistry. For the first 6 hours in culture, no significant change in the number of positive cells was seen. This was followed by a large increase in positive cells over the next 8 hours and a slow increase thereafter to 24

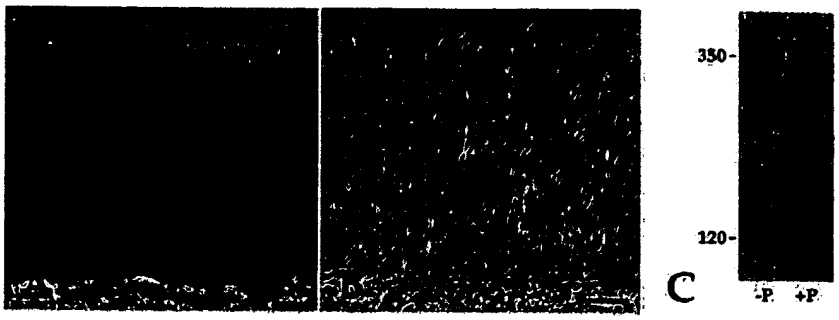


Fig. 3. Expression of *Notch* in the chick retina. (A) E4 chick retina after in situ hybridization with a digoxigenin-labelled antisense *CNotch-1* probe, with detection by alkaline-phosphatase-coupled secondary antibody. Pigmented epithelium is at the bottom, developing ganglion cell layer at the top. Bar, 100 μm. (B) Adjacent section of E4 chick retina, after in situ hybridization with a digoxigenin-labelled sense *CNotch-1* probe. (C) Western blot of E4 chick retinal extract, stained with a polyclonal antiserum raised against peptides from the cdc10/ankyrin repeat region of TAN-1, the human Notch homologue. –P, antibody without blocking peptide; +P, antibody preabsorbed with TAN-1 peptides before staining.

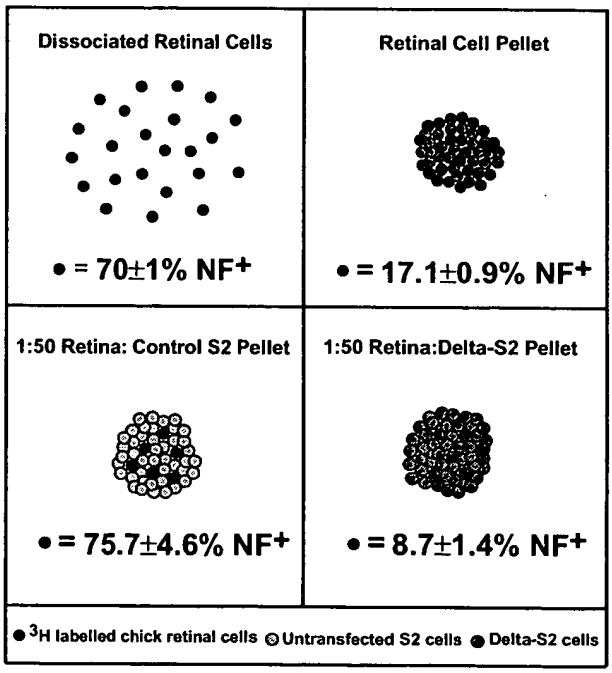


Fig. 4. Differentiation of chick retinal cells in pellets with S2 cells expressing Delta. E4 chick retinal cells were labelled as explants with [^{3}H]thymidine, then dissociated and cultured in pellets with a 50-fold excess of *Drosophila* S2 cells that had been transfected with a full-length *Delta* cDNA (Fehon et al., 1990), or control untransfected S2 cells, for 24 hours. Pellets were dissociated and cells stained for NF and developed for autoradiography, and the percentage of $^3H^+$ cells that were NF^+ counted.

hours (Fig. 1B). Cells maintained in culture for another 48 hours showed no further change in the percentage of cells expressing NF or RA4 (data not shown).

To assess whether the expression of NF in dissociated culture was specific to progenitors from the period of ganglion cell genesis in vivo, progenitors from different age embryos were labelled with [^{3}H]thymidine for 1 hour to mark cycling cells. Cells were then placed in dissociated cultures at the lowest density for 24 hours, followed by NF immunocytochemistry and autoradiography. The percentage of [^{3}H]thymidine-labelled cells that were also NF$^+$ after 24 hours in culture in this set of experiments increased from 13.2% at E2 to a maximum of 63.9% at E4 and decreased thereafter to 3.6% at E7 (Fig. 1C). To determine if the difference in differentiation reflected an intrinsic property of cells from different ages or a changing environment, dissociated cultures were made with [^{3}H]thymidine-labelled cells mixed with a 20-fold excess of unlabelled cells from a different age; in each case the percentage of [^{3}H]thymidine-labelled cells that expressed NF or RA4 was the same as the percentage when the labelled cells were cultured alone (data not shown). These results suggest that the competence of progenitors to differentiate into ganglion cells is correlated to the period of ganglion cell genesis in vivo.

Confirmation that the cells recognized by the NF and RA4 markers were ganglion cells was obtained in two ways. First, most NF$^+$ or RA4$^+$ cells were found to have morphological characteristics consistent with ganglion cell identity; they had a large cell soma and a single unipolar process many cell diameters long with a terminal enlargement resembling a growth cone (see Fig. 2). Second, eight other ganglion-cell-specific antibodies, directed against other neurofilament epitopes, neurofilament-associated proteins, α_6-integrin, TrkB, NgCAM and Gap-43 (see Materials and Methods), were used to stain E4 (stage 24) retinal cells before and after 24 hours in low density culture and all showed a substantial increase over the 24-hour culture period (data not shown). These results suggested that the majority of early chick retinal progenitors were competent to differentiate as ganglion cells.

In order to begin to characterize the activity(s) responsible for the normal limitation of ganglion cell differentiation, co-culture experiments were done. Explants were co-cultured in a tissue culture well with dissociated cultures at the lowest density in collagen gels. No difference was seen in the number of NF$^+$ or RA4$^+$ cells in explants or dissociated cell cultures compared to each cultured alone, demonstrating that the inhibitory activity was not freely diffusible.

Notch-1 is expressed in the early chick retina

As the previous results indicated that an inhibitory activity that was not freely diffusible normally limited ganglion cell differentiation, we took a candidate molecule approach focusing on membrane-bound inhibitors of differentiation. As Notch had been shown to play such a role in *Drosophila*, we wondered if it might be the activity. To investigate this possibility, in situ hybridizations using a chicken *Notch-1 (CNotch-1)* probe were performed. These revealed *CNotch-1* expression in a gradient across the proliferative zone of the E4 retina, highest at the ventricular surface, but no expression in the differentiated ganglion cell layer (Fig. 3A,B). As the only differentiated cells at this age are ganglion cells (Prada et al., 1991), this suggested that *CNotch-1* is expressed in all undifferentiated cells of the early retina. Western blots on E4 chick retinal extracts, using an antibody to the human Notch homologue, TAN-1 (Fig. 3C), showed two bands of the predicted relative molecular mass (350 and 120×10^3 M_r), with the same relative mobilities as the TAN-1 protein in its full-length and processed forms (Aster et al., 1994).

The inhibitor of ganglion cell differentiation is mimicked by Delta

If Notch activity was responsible for the inhibition of ganglion cell differentiation in pellet cultures, then replacement of retinal cells with cells bearing a Notch ligand should result in inhibition of ganglion cell differentiation. To test this possibility, we co-cultured *Drosophila* S2 cells transfected with a full-length *Delta* cDNA, or untransfected S2 control cells (Fehon et al., 1990), with chick retinal cells in a pellet. To label the chick cells, E4 (stage 24) retinae were explanted into [^{3}H]thymidine-containing medium for 2 hours. They were then dissociated and mixed in a pellet culture with either untransfected S2 cells or S2-Delta cells in a 1:50 ratio. Pellets were cultured for 24 hours, dissociated, stained for NF and processed for autoradiography. The results (Fig. 4) demonstrate several points. First, absence of cell-cell contact is not necessary to induce differentiation, because a high degree of ganglion cell differentiation was observed in retinal cells in contact with untransfected S2 cells. This suggests that one or more specific activities allowed by cell-cell contact inhibits ganglion cell differentiation. Second, *Drosophila* Delta protein, or another factor induced by Delta, is capable of inhibiting differentiation of chick retinal progenitors into ganglion cells, to an even greater degree than other retinal cells. Third, interspecies conservation of Delta between *Drosophila* and any putative chick homologue is sufficient to allow functional substitution, at least in this assay. This result is consistent with the finding of conservation of binding of Delta to *Xenopus* Notch EGF repeats 11-12 in an in vitro assay (Rebay et al., 1991). These data support a role for Notch in ganglion cell differentiation and together with recent data that a *Delta* homologue is expressed in the embryonic chick retina (D. Henrique and J. Lewis, personal communication) suggest that Delta may be the Notch ligand operative in the inhibition of ganglion cell differentiation.

Inhibition of *Notch* expression increases retinal ganglion cell number

These experiments suggested that the reason 70% of retinal cells differentiate as ganglion cells in low density culture is that they are relieved from inhibition by dissociation of Notch from its ligand(s). A decrease in Notch activity in the intact chick retina should then result in an increase in differentiation of ganglion cells. Antisense oligonucleotides were used to test this hypothesis. Phosphothiorate-modified 23-mer antisense oligonucleotides were designed to three regions of the *Notch* mRNA: the EGF repeat region (oligonucleotide designated EGFR), the lin-12/Notch repeat region (designated LNR) and the cdc10/ankyrin repeat region (designated CDCR) (see Fig. 6A). E2 (stage 16) chick eyes were injected with each of these oligonucleotides, with harvest at E5 (stage

27). Retinae were fixed, sectioned and stained with a monoclonal antibody to the Islet-1 protein (Yamada et al., 1993) as a marker of ganglion cells. This antibody is ganglion cell-specific throughout chick retinal development (C. P. A. and C. L. C., unpublished). Harvest was on E5, rather than later in development when other cell types are present, in order to examine the effect of a change in Notch level specifically on ganglion cell differentiation. By E5, the majority of ganglion cells have been born, but other cell types have not (Prada et al., 1991).

The thickness of the ganglion cell layer in the antisense-injected retinae was approximately twice that of the sense-injected control retinae (Fig. 5). The shape, size and total number of cells in the retina did not change with any oligonucleotide injection, however, and there were no ectopically located Islet-1$^+$ cells. Staining with NF and RA4 confirmed the maintenance of approximately normal retinal architecture, although Nomarksi optics revealed that the retina appeared less tightly organized and the individual cells had a slightly irregular shape (Fig. 5). Quantitation of Islet-1$^+$ cells on sections showed a graded increase in ganglion cell layer thickness from center to periphery, reflecting the spatial gradient of development of the retina and the fact that the peripheral retina at this stage normally has very few Islet-1$^+$ cells (Table 1). No change was seen in the contralateral eye of injected embryos, or in sense-injected eyes, relative to uninjected controls.

Similar results were obtained by explanting whole E4 retinae into medium containing 25 μM oligonucleotide and culturing for 24 hours. Following the culture period, the explants were dissociated, fixed and stained for NF or RA4. Explants cultured without oligonucleotide showed a small increase in the number of positive cells during the culture period (Fig. 1A). In the presence of 25 μM sense oligonucleotide, there was a similar small increase in the number of positive cells, which was not significantly different from the no oligonucleotide condition. In the presence of 25 μM antisense oligonucleotide, however, there was a 66-80% increase in the percentage of cells staining for NF or RA4, relative to the no oligonucleotide condition (Fig. 6B). All three oligonucleotides gave qualitatively similar results and addition of the three oligonucleotides together at a concentration of 25 μM gave no further increase in positive cells. A dose-response was seen in these effects, such that at 0.1 or 1 μM, no change was seen; at 5 μM, a 25-30% increase in NF$^+$ or RA4$^+$ cells was seen and at 100 μM, there was evidence of toxicity.

That three antisense sequences directed against different regions of the *Notch* mRNA had the same effect suggested that the oligonucleotide effect was specific to the *CNotch-1* target mRNA. This specificity was tested further by introducing nucleotide mismatches into the lin-12/Notch repeat (LNR) oligonucleotide sequence and asking what effect these mismatches had on the observed antisense effect. LNR oligonucleotides were synthesized with one, three or five nucleotide mismatches introduced at regular intervals along the sequence and E4 retinal explants were incubated in a 25 μM concentration of each for 24 hours. The efficacy of the antisense oligonucleotide in raising the percentage of NF$^+$ cells decreased in a stepwise fashion with increasing sequence mismatches, so that the oligonucleotide with 5 mismatches (M5) gave almost the same percentage of NF$^+$ cells as the sense oligonucleotide (Fig. 6C). The effect on ganglion cell number declined both with decreased length of maximum complementary sequence and with decreased cumulative identity of the sequence, arguing that both appear to be important in determining the effectiveness of antisense oligonucleotides in bringing about specific degradation of their target RNAs, consistent with the conclusion reached by Woolf et al. (1992).

CNotch-1 mRNA in the oligonucleotide-treated explants was quantified by RNase protection (Fig. 7). Similar decreases in *CNotch-1* mRNA were seen with each antisense oligonucleotide, whereas no change was seen with any of the sense oligonucleotides (Table 2). Several attempts were made to quantify a decrease in Notch protein levels using Western blots and ELISA assays. These were unsuccessful due to problems in reproducibly quantifying Notch protein levels even in untreated, freshly excised intact retinae.

The effect of antisense oligonucleotides on the proliferative activity of retinal cells was assessed by the inclusion of [^{3}H]thymidine for the duration of the explant culture period. After 24 hours, the explants were dissociated and the percentage of all cells that were ^{3}H-positive was counted in antisense- and sense-treated explants. In three separate experiments, the percentage of cells that were ^{3}H-positive was identical between sense-treated (51.2±2.3%) and antisense-treated (50.1±1.9%) retinae.

Expression of constitutively active *Notch* decreases ganglion cell number

If the hypothesis that *Notch* limits ganglion cell differentiation is correct, expression of a constitutively active form of Notch in the early retina should decrease ganglion cell production, if Notch activation is normally limiting. The intracellular domain of Notch, with or without the transmembrane domain, has been shown to be constitutively active (Rebay et al., 1993; Struhl et al., 1993, Lieber et al., 1993). We therefore constructed a retroviral vector encoding the intracellular domain of the human *Notch* homologue, *TAN-1*, and tested its effect on ganglion cell production.

The replication-competent retroviral vector TANIC carried the intracellular 2362 bp of *TAN-1*, encoding a protein starting 13 amino acids intracellular to the transmembrane domain and continuing to the C terminus. E2 (stage 16) chick retinae were infected in vivo with TANIC or a control vector carrying the human placental alkaline phosphatase gene (vector APA; Fekete and Cepko, 1993). To assess the effect of TANIC infection on ganglion cell production in vivo, infected retinae were harvested at E5 (stage 27) and sectioned. To identify infected cells, sections were processed for viral capsid protein (p27 gag) immunohistochemistry and only heavily infected (p27$^+$) areas of the retinae were examined. Ganglion cells were visualized on sections by Islet-1 immunohistochemistry.

Infection of the retina was widespread at E5, as assessed by p27 staining. In p27$^+$ areas of TANIC-infected retinae, the ganglion cell layer was thinned to half its normal width (Fig. 8). The ganglion cell layer was several cells thick in p27$^-$ areas of TANIC-infected retinae and in APA-infected control retinae. In contrast, in p27$^+$ areas of TANIC-infected retinae, the ganglion cell layer was generally one cell thick, with some

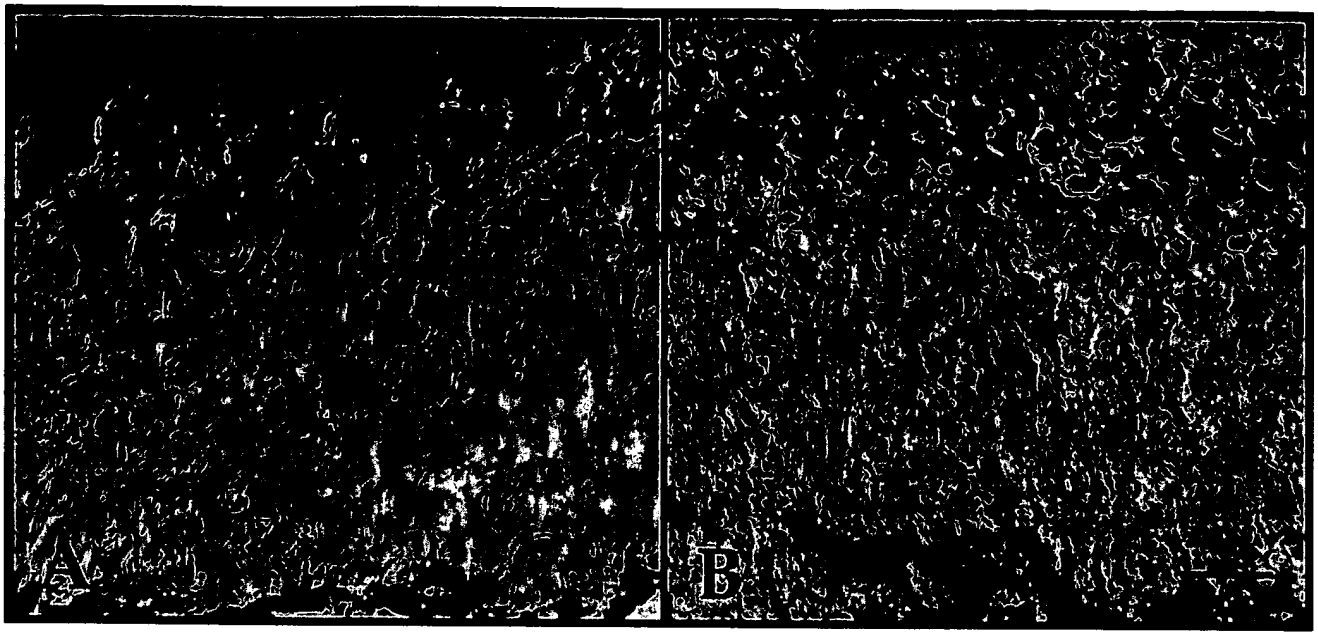


Fig. 5. Effect of *Notch* antisense oligonucleotide in vivo. Eyes of chick embryos were injected with either sense or antisense oligonucleotides at E2 and harvested at E5. Sections were stained with a monoclonal antibody to the Islet-1 protein as a marker for ganglion cell identity, using horseradish peroxidase coupled secondary antibodies for detection. Pigmented epithelium is at the bottom and developing ganglion cell layer at the top. (A) Eye injected with CDCR-sense oligonucleotide. (B) Eye injected with CDCR-antisense oligonucleotide (see text for definition of terms). Bar, 100 μm.

Table 1. Change in islet 1$^+$ cell number in retinae after antisense injection

Region of retina	CDCR*	LNR†	EGFR‡
Central	+108±14%§	+76±7%	+74±6%
Intermediate	+167±36%	+108±10%	+94±12%
Peripheral	+1019±58%	+481±29%	+341±26%

*Oligonucleotide from rat *Notch-1* in cdc10/ankyrin repeat region
†Oligonucleotide from chick *Notch-1* in lin-12/Notch repeat region
‡Oligonucleotide from chick *Notch-1* in EGF repeat region
§Mean±s.e.m. of percent increase in Islet 1$^+$ cells in antisense- versus sense-injected retinas (n=5 for each, P<0.01 for each comparison).

areas being devoid of ganglion cells altogether. Quantitation of Islet-1$^+$ cells was done by the same method as was used with the oligonucleotide-treated retinae. The number of Islet-1$^+$ cells in TANIC-infected retinae was decreased in a center-to-periphery gradient consistent with the pattern of neurogenesis in the retina (Table 3).

Infection with TANIC had no effect on the proliferative activity of retinal cells. This was assessed by infecting with TANIC on E2 and either injecting the eye with [^{3}H]thymidine or harvesting retinae as explants into medium containing [^{3}H]thymidine at E4, with harvest 1 day later. Both sets of retinae were then dissociated and the cells stained for p27 and then processed for autoradiography. Results with each technique were similar. The percentage of cells that were labelled with [^{3}H]thymidine was no different among uninfected (41.4±2.1%), APA-infected (44.3±3.3%) and TANIC-infected (42.5±3.1%) cells.

The fact that 70% of cells in the E4 retina differentiated as ganglion cells in low-density dissociated cell culture or in a co-pellet with S2 cells, but not in a co-pellet with Delta-S2 cells, suggested that inactivation of Notch signalling by separation of Notch from its ligand was the critical change that caused differentiation in dissociated culture. This model predicts that cells expressing constitutively active Notch should be relatively insensitive to the effects of dissociation. To test this, retinae were infected with TANIC or APA at E2 and dissociated at E4. Some cells were processed immediately for p27 and NF immunocytochemistry, while other cells were cultured at the lowest density for 16 hours, then stained for p27 and NF. The effect of viral infection on ganglion cell differentiation was assessed by comparing NF immunoreactivity in virally infected (p27$^+$) and uninfected (p27$^-$) cells from the same retina. Prior to culture, the percentage of uninfected (p27$^-$) cells that were NF$^+$ in TANIC retinae was 14.2±2.1%, consistent with previous results (Fig. 1B). However, only 7.8±0.8% of the p27$^+$ cells were NF$^+$ in these retinae (a 45% decrease, P=0.02) (Fig. 9A). This decrease was similar to the decrease in Islet-1$^+$ cells seen on sections (Table 3). In APA control-injected retinae, the percentage of uninfected and infected cells that were NF$^+$ at the start of the cultures was the same (11.7±2.3% of the uninfected cells, versus12.3±2.2% of infected cells, P>0.10). Similar results were seen after staining with RA4 (data not shown). The cells were then cultured at low density in collagen gels for 16 hours. At the end of this period, the percentage of uninfected (p27$^-$) cells that were NF$^+$ in TANIC-infected retinae was 58.2±2.5%, consistent with previous data (Fig. 1B), while the percentage of infected (p27$^+$) cells that were NF$^+$ was decreased 40%, to 34.9±3.4% (P=0.01) (Fig. 9A). Infection with APA had no effect on the percentage of cells expressing NF (Fig. 9A).

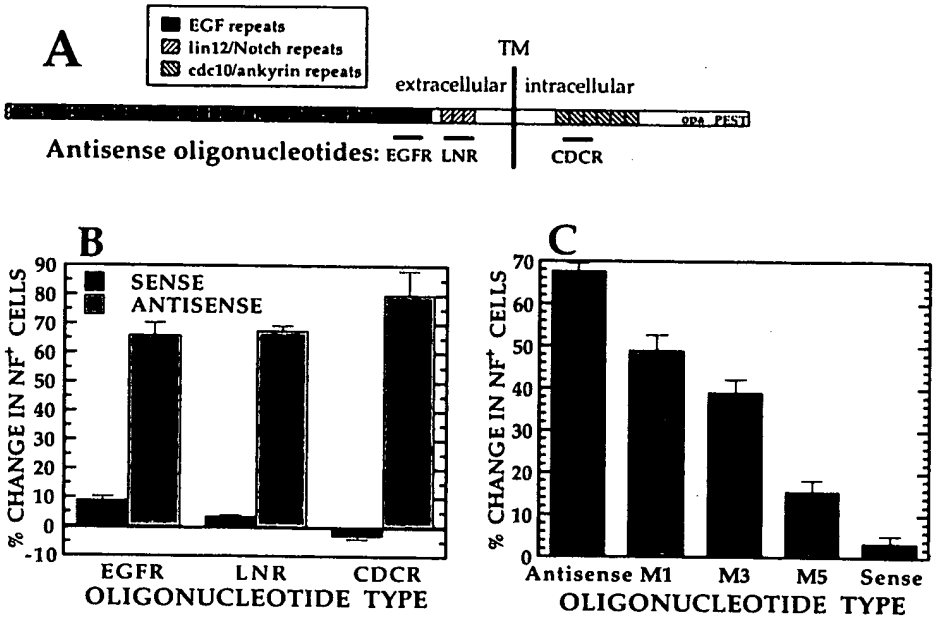


Fig. 6. Effect of Notch antisense oligonucleotides on ganglion cell differentiation in explants. (A) Schematic representation of the proteins encoded by *Notch*, with locations of oligonucleotides directed against the corresponding mRNA (oligonucleotide sizes not to scale). All Notch homologues display a conserved structure. They are transmembrane proteins with an extracellular domain containing 36 epidermal growth factor-like repeats (EGFR), followed by three *lin-12/Notch* repeats (LNR). The cytoplasmic domain contains six cdc10/ankyrin repeats (CDCR), a polyglutamine (opa) sequence and a putative PEST sequence (Wharton et al., 1985; Kidd et al., 1986). Antisense oligonucleotides were directed against a sequence in each of the three conserved repeat domains. (B). E4 (stage 24) retinal explants were cultured for 24 hours with no oligonucleotide, or in the presence of 25 μM oligonucleotide, then dissociated and stained for neurofilament (NF). Oligonucleotides are named as in A. Results are expressed as the percent change in NF^+ cells in oligonucleotide-treated explants compared to explants cultured without oligonucleotide. (C) Nucleotide mismatches decrease the ability of the LNR antisense oligonucleotide to increase numbers of NF^+ cells in explant cultures. Methods as in B. M1=1 mismatch, sequence 5′-CCAGCACTGCAΔTGACTGTGAGC-3′; M3=3 mismatches, sequence 5′-CCAGCGCTGCAΔTGACTΔTGAGC-3′; M5=5 mismatches, sequence 5′-CCAGCGCTΔCAΔTGGCTΔTGAGC-3′(mismatches underlined).

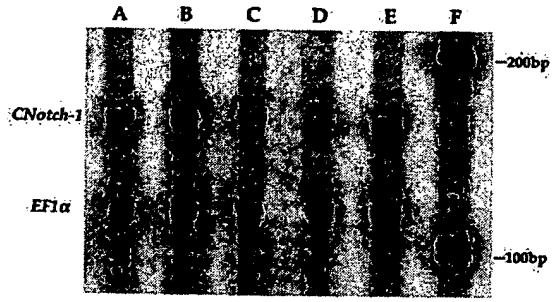


Fig. 7. RNase protection of *Notch* mRNA in oligonucleotide-treated explants. To measure a specific decrease in *CNotch-1* mRNA, the total RNA from treated explants was analyzed for the amount of *CNotch-1* and *EF1α* RNA using RNase protection. *CNotch-1* probe and *EF1α* control probe were included in the same tube and 5 μg retinal RNA were used for each reaction. (A) No oligonucleotide treatment; (B) CDCR-sense oligonucleotide; (C) CDCR-antisense oligonucleotide; (D) LNR-antisense oligonucleotide; (E) EGFR-antisense oligonucleotide; (F) RNA molecular weight markers.

Table 2. Change in *CNotch-1* mRNA level after oligonucleotide treatment

Oligonucleotide type	Change in *CNotch-1* mRNA level*
CDCR antisense	−28.1±4.5%
LNR antisense	−32.0±9.4%
EGFR antisense	−24.7±5.3%
CDCR sense	+5.2±7.2%
LNR sense	+1.2±5.1%
EGFR sense	−2.9±3.5%

**CNotch-1* mRNA was measured by RNase protection with an *EF1α* internal control and expressed as the difference in mRNA level between oligonucleotide treated and control retinae.

As a control for the specificity of the TANIC sequence in bringing about inhibition of ganglion cell differentiation, parallel dissociated cultures were made with TANIC-infected retinal cells, but with the addition of 25 μM antisense CDCR oligonucleotide in the culture media. This treatment would be expected to bring about degradation of both TANIC and endogenous *CNotch-1* mRNA. Addition of CDCR antisense oligonucleotides to dissociated cultures eliminated the effect of TANIC infection on NF expression, restoring the percentage of $p27^+$ cells that were NF^+ to control levels (Fig. 9B).

DISCUSSION

In the first differentiation event of retinal development, ganglion cells arise from a neuroepithelium devoid of other differentiated cell types. The work presented here shows that this occurs by selection from a larger group of progenitors with competence to produce ganglion cells, through the action of Notch. On the basis of these findings, we suggest that the majority of the early chick retina may constitute an equivalence group, the primary fate of which is the first born neuron in the retina, the ganglion cell. A similar suggestion has been made concerning the first born neuron in the *Drosophila* retina, R8 (Cagan, 1993). An equivalence group has been defined as a group of cells that have a common developmental potential, but which normally acquire different fates as a result of cell-cell interactions (Wigglesworth, 1940; Kimble et al., 1979). Initially described in nematodes, neural equivalence groups have been described in multiple invertebrate species

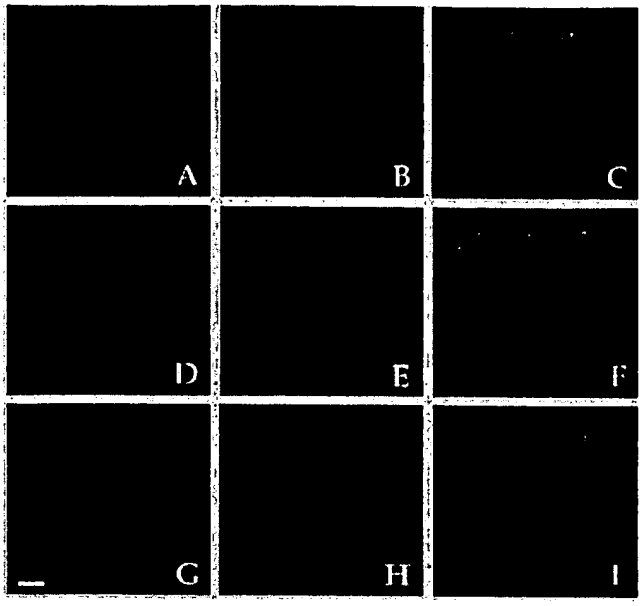


Fig. 8. Effect of infection with TANIC on ganglion cell differentiation in vivo. Retinae were infected with TANIC or APA control virus at E2 (stage 16) and harvested at E5 (stage 27). Sections were stained with DAPI to show cell nuclei (A,D,G), antibody to p27 gag protein as a marker of viral infection with FITC-conjugated secondary antibody (B,E,H), and antibody to the Islet-1 protein as a marker of ganglion cell identity with Texas-Red-conjugated secondary antibody (C,F,I). Top row (A-C) uninfected retina; middle row (D-F) retina infected with APA virus; bottom row (G-I) retina infected with TANIC virus. Quantitation of these data are in Table 3. Bar, 100 μm.

Table 3. Change in Islet 1$^+$ cell number in retinae after TANIC infection

Region of retina	% Decrease versus APA
Central	−41.3±2.6%*
Intermediate	−45.9±3.1%
Peripheral	−93.8±9.4%

*Difference in mean±s.e.m. (fields counted/region/retina=5; retina n=5) of Islet-1$^+$ cells in p27 gag$^+$ regions of retinae.

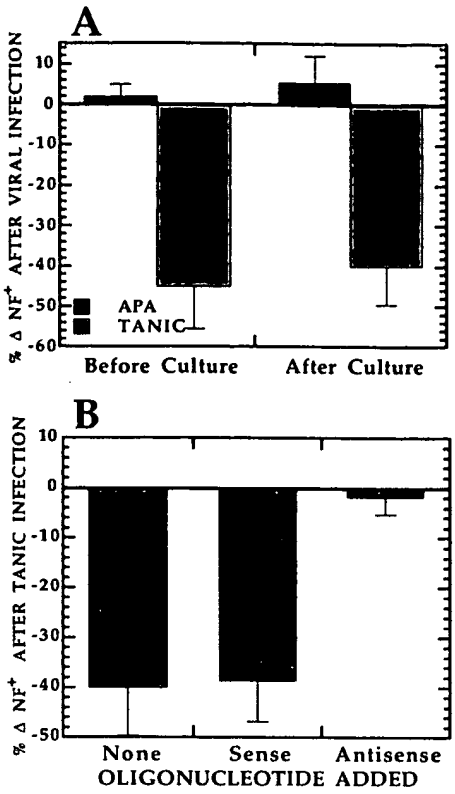


Fig. 9. Infection with TANIC decreases ganglion cell development in vitro. (A) The ability of cells to express NF in low density collagen gel cultures after infection with TANIC or the control APA virus was assessed. Retinae were infected in vivo with TANIC or APA at E2 (stage 16) and harvested at E4 (stage 24). Retinae were dissociated and either stained immediately for p27 gag and NF, or cultured for 16 hours at 0.25×10^5 cells/25 μl in collagen gels prior to staining. Uninfected (gag$^-$) and infected (gag$^+$) cells from each retina were assayed for whether they expressed NF. Results are plotted as the percent difference in NF$^+$ cells between uninfected cells and cells infected with APA or TANIC. (B) Antisense oligonucleotides directed against the intracellular domain of *Notch* eliminate the effect of TANIC in dissociated cultures. Techniques as in A, except that 25 μM CDCR sense or antisense oligonucleotide was added to some of the cultures.

(Muskavich, 1994; Taghert et al., 1984; Shankland and Weisblat, 1984) and in zebrafish (Eisen, 1992). In *Drosophila*, neuroblast specification in the embryonic ectoderm (Doe, 1992) and specification of the sensory mother cells of the sensory macrochaetae (Simpson and Carteret, 1990), both occur from well-defined proneural equivalence groups. Though we have shown that the majority of retinal progenitors have the competence to differentiate as a single cell type, we have not shown that the alternative fates that these cells may acquire are also equivalent. Support for the existence of a ganglion cell equiv-alence group will require these data and these experiments are now in progress.

The competence of the remaining 30% of cells remains to be defined. This is the percentage of cells that remain mitotic at the end of the culture period (C. P. A. and C. L. C., unpublished data), so ongoing mitosis may prevent differentiation of these cells. It is also possible that this 30% of progenitors do not have the competence to differentiate as ganglion cells, or that all progenitors may not be competent simultaneously. The center-to-periphery gradient of retinal development (Prada et al., 1991) might indicate that the population that was cultured was heterogeneous, though culturing central and peripheral retinal cells separately did not increase the percentage of differentiated cells (data not shown). Technical reasons may also explain why all cells did not differentiate in the lowest density dissociated cultures. Some cells migrated into clumps during the culture period, allowing residual cell-cell contact; if this was the case, Notch does not appear to have been operative, because addition of Notch antisense oligonucleotides to dissociated cell cultures did not change the percentage differentiating as ganglion cells (data not shown). In addition, it remains possible that a non-Notch inhibitory factor was still active in the lowest density dissociated cultures, or that an inducer of ganglion cell differentiation was limiting.

Notch activity regulates ganglion cell number

Members of the *Notch/lin-12* family encode transmembrane proteins that are thought to act as receptors for lateral inhibitory interactions influencing cell fate choice among cells in equivalence groups in invertebrates (see Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995 for recent reviews). *Notch* homologues have been found in *Xenopus*, human, rat, mouse and zebrafish (Coffman et al, 1990; Ellisen et al., 1991; Weinmaster et al., 1991,1992; Franco del Amo et al., 1992; Bierkamp and Campos-Ortega, 1993; Lardelli et al., 1994). Chicken *Notch-1 (CNotch-1)* was found to be expressed in all undifferentiated cells in the retina where cell fate decisions were being made, in a gradient with the highest expression in the ventricular zone where cytokinesis takes place (Sidman, 1961). This expression pattern is similar to that reported for mouse *Notch-1* (Guillemot and Joyner, 1993).

The experiments using antisense oligonucleotides are the first demonstration of the effect of a tissue-specific decrease in Notch activity during vertebrate development. Differentiation of progenitors into ganglion cells was increased by antisense treatment, doubling the thickness of the ganglion cell layer in the central retina and precociously extending the region of the peripheral retina in which ganglion cell differentiation occurs. Absence of normal differentiation in the presence of activated Notch has led to the suggestion that Notch signalling inhibits differentiation (Coffman et al., 1993). Our results show directly that ongoing Notch activity is required to prevent competent cells from undergoing differentiation in vivo. The only previous report of a decrease in vertebrate Notch function described mouse *Notch-1* mutants created by homologous recombination (Swiatek et al., 1994). In homozygotes, there was widespread cell death and embryonic lethality before neuronal differentiation in the CNS took place. The neural tube and optic vesicle, both of which normally express *Notch-1* and are entirely proliferative at this stage, appeared normal up to E9 in the *Notch-1* knockout, suggesting that *Notch-1* activity is not required for the partitioning of neural from non-neural ectoderm, nor for early proliferation events in the mouse CNS. Consistent with this finding, we observed no change in the proliferation of chick CNS retinal progenitors after treatment with Notch antisense oligonucleotides.

Though the antisense oligonucleotides caused a decrease in *CNotch-1* mRNA level of only 25-30%, this relatively small decrease led to a doubling in ganglion cell number, suggesting that progenitors are sensitive to small changes in Notch activity. This conclusion is consistent with (1) the effects of small changes in gene dosage of either *Notch* or *Delta* in *Drosophila* (Artavanis-Tsakonas, 1988; Muskavitch, 1994), (2) the suggestion that differentiation of cells within a proneural equivalence group occurs in response to small random variations in Notch level (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991) and (3) the fact that such small initial variations may be amplified by subsequent effects on expression of both ligand and receptor (Wilkinson et al., 1994). All of these data are supportive of the idea that the Notch protein level is actively regulated, with a short half-life (Aster et al., 1994), as had been suggested by the presence of a PEST sequence, typical of rapidly degraded proteins (Rogers et al., 1986). It is also possible that the RNase protections underestimated the effect on Notch protein levels, as we were unable to reliably measure Notch protein; alternatively, *CNotch* RNA level may have been decreased to a greater extent in the subpopulation of cells that differentiated.

While the antisense oligonucleotide treatment led to a doubling in the number of ganglion cells, to an absolute level of 40%, dissociated culture led to 70% of the cells becoming ganglion cells. This difference could be explained by a failure of the oligonucleotides to reduce *CNotch-1* mRNA to a greater degree. Alternatively, it is possible that an additional inhibitory signal is responsible for the submaximal ganglion cell differentiation in explants, but is inactivated in the dissociated cultures along with Notch.

Infection of retinal progenitors early in the period of ganglion cell generation with a retrovirus (TANIC) encoding the intracellular domain of the human Notch homologue (TAN-1) produced the opposite effect to that seen after antisense oligonucleotide injection. In the central retina, the number of ganglion cells was decreased by 40% and in the peripheral retina by 90%. This effect on differentiation was cell autonomous, affecting only those cells expressing viral antigens, consistent with the prevailing model of Notch as a receptor of cell-fate-determining signals (Artavanis-Tsakonas et al., 1995). In addition, progenitors expressing activated Notch were resistant to the effects of dissociation on ganglion cell differentiation, as would be expected if the reason for differentiation in dissociated culture was inactivation of Notch by dissociation from its ligand. The fact that the control level of ganglion cell differentiation could be restored by incubating TANIC-infected dissociated cells with Notch intracellular domain antisense oligonucleotide was somewhat surprising, given the partial effect on differentiation seen in the other experiments. This finding suggests that the viral RNA was either low in amount and/or more sensitive to antisense degradation.

Some progenitors may be insensitive to Notch signalling, because consitutive activation of Notch with TANIC, or supplying excess ligand in the Delta-S2:retinal cell co-pellet assay, decreased the number of ganglion cells by half, but did not eliminate them. Determining the mechanism by which cells escape Notch inhibition in these assays should help answer the question of how cells escape Notch inhibition during normal development. Stochastic fluctuations in Notch level have been hypothesized to lead to differentiation (Seydoux and Greenwald, 1989; Heizler and Simpson, 1991), but this cannot explain the results with TANIC. It is unlikely that sequence divergence of *CNotch-1* from *TAN-1* was responsible for TANIC resistance, as *Notch* homologues are highly conserved in this region. Some cells that expressed the viral p27 gag protein may not have expressed TAN-1, though expression of gag is highly correlated with expression of the nonviral gene (Fekete and Cepko, 1993); we were unable to address this directly because our antibodies did not detect Notch protein on sections. Alternatively, because Notch may be a multifunctional receptor, with activation by different ligands leading to distinct effects on cell fate (Couso and Martinez Arias, 1994; Artavanis-Tsakonas et al., 1995), ligand-independent perturbations of Notch activity such as were done here may have altered multiple Notch pathways simultaneously, resulting in an intermediate phenotype. Some ganglion cells would be expected after in vivo infection because, at the time of infection, 20% of ganglion cells were

postmitotic (Prada et al., 1991) and thus resistant to retroviral infection. More complete TANIC infection was expected in the retinal periphery, because fewer cells were postmitotic there at the time of infection and greater suppression of ganglion cell production was indeed observed in the periphery (see Table 3). Finally, a Notch-independent ganglion cell-inducing signal may explain the ganglion cell persistence.

In the dissociated cell culture experiments, a characteristic of viral transduction may have led to the observed TANIC resistance. Because the virus was replication competent, viral spread occurred after the initial infection, so infected cells varied in their time of exposure to activated Notch. Viral spread continued to occur in the dissociated cultures, as demonstrated by a two-fold increase in the percentage of cells that stained for the viral gag protein during the culture period (data not shown). Given the short latency to ganglion cell differentiation in dissociated culture (see Fig. 1B), many cells that were infected with TANIC shortly before or during culture would have differentiated before viral expression occurred, so would show both gag and neurofilament expression at the end of the culture. This heterogeneity would be minimized by the use of a replication-incompetent viral vector carrying the same construct and these experiments are now in progress.

We do not know what the cells expressing TANIC that are diverted from the ganglion cell pathway eventually become, nor do we know at what cells' expense the extra ganglion cells are produced in the antisense experiments. All harvests were done at or before the E5, in order to examine effects specific to ganglion cell genesis. No other cell types are produced before E6 (Prada et al., 1991) and none express specific markers until E7 (C. P. A., C. L. C. and S. Bruhn, unpublished data), so it was not possible to assay for other cell types. This has made assessment of the equivalence of these cells' alternative fates difficult. Recent experiments in the *Xenopus* retina (Dorsky et al., 1995) suggest that most cells transfected with an intracellular domain *Xotch* mRNA (*XotchΔE*) do not immediately switch into another cell fate but remain undifferentiated, at least temporarily. A related question is whether Notch plays a role in the genesis of later-born cell types in the chick retina. *Notch* is required for the correct differentiation of multiple cell types in the *Drosophila* retina (Cagan and Ready, 1989; Fortini et al., 1993) and *CNotch-1* was observed to be expressed in the chick retina by in situ hybridization throughout the period of generation and differentiation of every retinal cell type (data not shown). It is therefore likely that Notch plays a role in other cell fate decisions in the retina and preliminary results suggest that this is so in the postnatal rat retina (Z. Z. Bao and C. L. C., unpublished data).

Lastly, multiple aspects of this work suggest that perturbations of Notch activity do not affect mitotic behavior of progenitor cells. The gross morphology, size and cell number of retinae injected with antisense oligonucleotides or TANIC were the same as in control retinae and labelling with [^{3}H]thymidine demonstrated directly that mitotic activity was not affected by these perturbations and suggested that Notch antisense effects were restricted to postmitotic cells. Consistent with this hypothesis, Coffman et al. (1993) found that the increase in neural and muscle tissue seen after injection of blastomeres with *XotchΔE* mRNA was unaffected by inhibition of cell division by aphidicolin and hydroxyurea and Dorsky et al. (1995) reported no BrdU incorporation beyond what is normally observed in a small number of *XotchΔE*-transfected retinal cells.

Competence, Notch and acquisition of vertebrate neuronal cell fate

A working model for cell fate acquisition in vertebrate retinal development can be suggested based on these and related data. Cell lineage studies have shown that vertebrate retinal progenitors are capable of producing multiple cell types throughout development (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). Cell birthdating studies (e.g., Prada et al., 1991) have shown discrete periods in development during which each cell type is produced and differentiation of progenitors in culture reflects these birthdays (Reh and Kljavin, 1989; Adler and Hatlee, 1989). Intrinsic properties of progenitors have been suggested to at least partially control when different cell types are produced, as early progenitors appear to have limited competence to produce later-born cell types (Watanabe and Raff, 1990). The competence of progenitors to produce a given cell type in the retina may therefore be acquired and lost in a temporal pattern mirroring the order of birth of the different cell types, and more progenitors may be competent to produce each cell type than normally do so in vivo. Cell fate acquisition in this model would be regulated in part by changes in progenitor competence and in part by selection from among this group by the action of environmental factors. Competence may be mediated by the action of such genes as the *as-c* homologues proposed to be involved in acquisition of neuronal competence in vertebrates (e.g., *XASH-3* (Turner and Weintraub, 1994; Ferreiro et al., 1994). Selection may occur through the action of positive or negative environmental factors in concert with regulation of the Notch pathway, many members of which have been identified in vertebrates in addition to the *Notch* homologues themselves (Sasai et al., 1992; Lindsell et al., 1995; Matsunami et al., 1989; D. Henrique and J. Lewis, personal communication). Though Notch clearly acts to inhibit differentiation as shown in a number of in vivo and in vitro systems, and we have shown here that inhibition of Notch is sufficient to induce differentiation, its effects on cell fate may be more complex (Artavanis-Tsakonas et al., 1995). These events appear to be regulated independently from mitotic activity of progenitors, at least in early neurogenesis, because neither differentiation (Harris and Hartenstein, 1991) nor Notch-induced developmental events (this paper, Dorsky et al., 1995; Coffman et al., 1993) appear to require cell division.

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Ganglion cells influence the fate of dividing retinal cells in culture

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SUMMARY

The different retinal cell types arise during vertebrate development from a common pool of progenitor cells. The mechanisms responsible for determining the fate of individual retinal cells are, as yet, poorly understood. Ganglion cells are one of the first cell types to be produced in the developing vertebrate retina and few ganglion cells are produced late in development. It is possible that, as the retina matures, the cellular environment changes such that it is not conducive to ganglion cell determination. The present study showed that older retinal cells secrete a factor that inhibits the production of ganglion cells. This was shown by culturing younger retinal cells, the test population, adjacent to various ages of older retinal cells. Increasingly older retinal cells, up to embryonic day 9, were more effective at inhibiting production of ganglion cells in the test cell population. Ganglion cell production was restored when ganglion cells were depleted from the older cell population. This suggests that ganglion cells secrete a factor that actively prevents cells from choosing the ganglion cell fate. This factor appeared to be active in medium conditioned by older retinal cells. Analysis of the conditioned medium established that the factor was heat stable and was present in the <3 kDa and >10 kDa fractions. Previous work showed that the neurogenic protein, Notch, might also be active in blocking production of ganglion cells. The present study showed that decreasing *Notch* expression with an antisense oligonucleotide increased the number of ganglion cells produced in a population of young retinal cells. Ganglion cell production, however, was still inhibited in cultures using antisense oligonucleotide to *Notch* in medium conditioned by older retinal cells. This suggests that the factor secreted by older retinal cells inhibits ganglion cell production through a different pathway than that mediated by Notch.

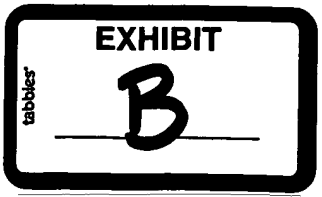
Key words: Chick, Determination, Induction, Notch, Ganglion cell, Retina

INTRODUCTION

The vertebrate central nervous system is composed of a great diversity of cell types. These cell types arise from a seemingly homogenous population of cells that makes up the neural tube. The fates of individual cells appear to be determined by a variety of mechanisms, which are poorly understood. Being composed of just seven major cell types, the neural retina is a valuable model used to study the mechanisms that determine the fate of cells in the developing nervous system. The retina arises as an outpocketing of the neural tube early in development (Dowling, 1987; Rodieck, 1973). The progeny of any one retinal progenitor cell in this outpocketing can differentiate into any one of the major cell types that comprise the mature retina (Turner and Cepko, 1987; Turner et al., 1990; Holt et al., 1988; Wetts and Fraser, 1988). Current evidence suggests that the fate of individual retinal cells is determined by a combination of cell-intrinsic mechanisms and of various cell-cell interactions, which are not yet fully characterized.

The ganglion cell phenotype appears to be the default state of all progenitor cells in the early developing retina. When retinal progenitor cells are allowed to differentiate isolated from other retinal cells in vitro, most of the cells differentiate as ganglion cells (Reh, 1992; Austin et al., 1995). Furthermore, ganglion cells start to differentiate within minutes following mitosis (Waid and McLoon, 1995), which suggests that the ganglion cell fate is inherited by cells rather than acquired during postmitotic events. Finally, the ganglion cell is one of the first cell types to be produced in vertebrate retina, and few ganglion cells are generated late in development (Rubinson and Cain, 1988; Snow and Robson, 1994; Belecky-Adams et al., 1996). These results suggest that cells will differentiate into ganglion cells in the absence of instructive signals from differentiated cells. The question remains as to why all retinal cells do not differentiate as ganglion cells. One possibility is that, as the first cells differentiate into ganglion cells, they express factors that actively inhibit production of more ganglion cells and promote determination of other cell types.

Several studies provide evidence that cell-cell interactions play a major role in determining cell fate (reviewed by Altshuler et al., 1991). Studies in which cells from early developing retina were cultured with cells from older retina showed an increased production of rod cells in the younger cell population than would have appeared if cells from the younger retina were cultured alone (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992). Rod cells are normally produced late in development (Carter-Dawson and LaVail, 1979; Altshuler et al., 1991). These findings can be interpreted to mean that



differentiated cells present in older retina, which would include ganglion cells, produce factors that promote development of late developing cell types. A similar result was obtained when a porous membrane that did not allow cells to touch separated the cells of the two ages. This indicates that the active factors are secreted and are soluble in the extracellular compartment. Several secreted factors have been identified that are expressed in developing retina and that promote development of the rod cell type. These include taurine, CNTF, bFGF and Sonic Hedgehog (Hicks and Courtois, 1992; Altshuler et al., 1993; Fuhrmann et al., 1995; Kirsch et al., 1996; Levine et al., 1997). A prediction suggested by these findings is that, in mixed cell cultures, while secreted factors released by older retinal cells promote development of late developing cell types in the young cell population, a concomitant reduction in production of early developing cell types, such as ganglion cells, should result.

In addition to findings that suggest a role for secreted factors, recent findings indicate that signaling through cell-cell contact mediated by Notch is involved in directing cell fate decisions. The *Notch* genes encode transmembrane, cell surface receptors that are involved in development of the *Drosophila* and vertebrate eye. In *Drosophila* and in vertebrates, the Notch proteins are activated by cell surface ligands, Delta and Serrate/Jagged (Arvantis-Tsakonas et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996). When expression of *CNotch-1* was blocked in early developing chick retina by application of antisense oligonucleotide, an increase was observed in the number of ganglion cells that developed and, conversely, when Notch was constitutively active, a decrease in ganglion cell production was observed (Austin et al., 1995). Similar results were obtained by manipulating expression of *CDelta-1* (Ahmad et al., 1997). One interpretation of this result is that activation of Notch by certain cell-cell contacts blocks cells from assuming the ganglion cell phenotype. This suggests the possibility that cell-cell-contact-mediated signaling is important in early cell fate decisions and that secreted factors are important in late phenotypic decisions. If this is the case, then it may be that factors that affect rod cell production have no effect on ganglion cell production.

The study reported here assessed the relative roles of secreted factors and Notch-mediated cell-cell signaling in regulation of ganglion cell production in the developing retina. The results showed that older retinal cells produce a secreted factor or factors that inhibit development of additional ganglion cells and that ganglion cells themselves may be a major source of the factor. The findings of this study also suggest that, among the factors regulating production of ganglion cells, the secreted factor acts at a higher level or in a separate pathway than does Notch.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs, pathogen-free White Leghorn crossed with Rhode Island Red, were obtained from the University of Minnesota Poultry Research Center. The eggs were incubated at 37°C in an egg incubator. Chicks with retinas depleted of ganglion cells were prepared using a technique described by Hughes and McLoon (1979). Briefly, the embryos were removed from the shells after 3 days of incubation and placed in culture chambers. The primordial optic tecta were destroyed by applying heat to the tectal surface by electrocautery. Cultured embryos with tectal ablations were maintained in a forced-draft tissue culture incubator at 37°C, 95% relative humidity and 1% CO_2. The retinas from some of the embryos were processed for immunohistochemistry with the RA4 antibody to confirm the loss of ganglion cells by E14.

Cell culture

The cell culture technique used in this study was adapted from Watanabe and Raff (1992). Embryonic day 4 (E4) chick retinas were dissected in culture medium and dissociated by gentle trituration. Cells were pelleted by centrifugation at 500 *g* for 4 minutes. This pellet, the test population, was placed on a polycarbonate membrane with a maximum pore size of 0.01 μm and covered with another membrane. Approximately 10^7 cells were used for each test cell pellet. A pellet of cells, the conditioning cell population, prepared the same way from E4, E7, E9, E14 or E14 ganglion cell-depleted retinas, was placed directly adjacent to the test cell population. Approximately three times the number of cells used in the test cell pellets was used in the conditioning cell pellet. The culture sandwich was maintained in F12 medium supplemented according to Bottenstein et al. (1980) with the addition of 2 mM glutamate and 1.5 μg/ml bromodeoxyuridine (BrdU). Cultures were incubated at 37°C and 5% CO_2 for 24 hours.

Immunocytochemistry

Cultures were fixed in 70% EtOH for 10 minutes and cryoprotected in 20% sucrose/phosphate buffer for 1 to 2 hours. The intact culture sandwiches were embedded in 10% tragacanth gum/20% sucrose/0.1 M phosphate buffer and sectioned at 10 μm with a cryostat. The sections were mounted on chrome alum/gelatin-coated glass slides. Sections were fixed to the slides with 1% paraformaldehyde in phosphate buffer for 2 minutes and rinsed in phosphate-buffered saline (PBS). DNA in the sections was denatured by incubation in 0.3 M NaOH for 2 minutes. The sections were rinsed in PBS and nonspecific antibody binding was blocked by incubation in 10% normal goat serum/ 0.3% Triton X-100/ PBS. These sections were incubated for 1 hour in an antibody to BrdU (1:10; Becton Dickinson), rinsed in PBS and incubated for 1 hour in goat anti-mouse IgG affinity-purified Fab fragment conjugated to fluorescein isothiocyanate (1.8 μg/ml; Jackson ImmunoResearch Labs Inc.). The sections were again rinsed in PBS and then incubated for 1 hour in goat anti-mouse IgG affinity-purified Fab fragment (80 μg/ml; Jackson ImmunoResearch Labs Inc.) to block any remaining binding sites. Sections were rinsed in PBS, incubated for 1 hour in an antibody to ganglion cells, RA4 (hybridoma culture supernatant; McLoon and Barnes, 1989), rinsed in PBS again and incubated for 1 hour in affinity-purified goat anti-mouse IgG conjugated to lissamine rhodamine B sulfonyl chloride (0.8 μg/ml; Jackson ImmunoResearch Labs Inc.). Following another rinse in PBS, sections were counterstained for 60 seconds with 1.5×10^{-6} μM DAPI. The stained sections were rinsed again in PBS and coverslipped with buffered glycerin mounting media (pH 7.0). These sections were viewed and photographed with an epiflourescence microscope, and the number of cells expressing particular labels was counted in the test cell populations. At least five fields were counted from each culture. At least five cultures were used for each datum point. All data are expressed as the mean ± s.e.m. The results of different experimental groups were compared using the Student's *t*-test.

Dying cells were identified in the sections of cultures using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, TUNEL, (Gavrieli et al., 92). Sections were rinsed in buffer and incubated for 1 hour at 37°C in digoxigenin-labeled UTP and TdT. Following three rinses, the sections were incubated for 30 minutes in an antibody to digoxigenin conjugated to fluorescein. Following further rinses, the sections were covered with a glass coverslip and SlowFade mounting media (Molecular Probes). Analysis was done as described above. In some cases, the same section after TUNEL analysis or adjacent sections were stained with Thionin. Pyknotic cells were counted in these sections as an indicator of cell death.

Conditioned medium

For conditioned medium studies, E9 chick retinas were dissociated and the cells were plated onto laminin-coated coverslips at a density of 1×10^5 cells/mm^2. Cultures were incubated at 37°C and 5% CO_2. After 24 hours, the medium was removed from the culture dishes, centrifuged at 500 *g* for 5 minutes and filtered through a low protein binding 0.22 μm PVDF membrane to remove cell debris. In some cases, the conditioned medium was size-fractionated prior to use with 3 kDa and 10 kDa Centriprep filters (Amicon), according to the manufacturer's instructions. Fresh medium was also size-fractionated by the same method as the conditioned medium and the corresponding missing fractions were added to the fractions of conditioned medium. This gave three differently sized fractions: >10 kDa, 3-10 kDa and <3 kDa. Immediately following preparation, conditioned medium was used to culture E4 retinal cell pellets as described above.

Antisense oligonucleotide

Antisense oligonucleotide was added to cultures to reduce expression of Notch. The sequence for the antisense oligonucleotide was taken from Austin et al. (1995). The oligonucleotide was designed to hybridize with the *lin-12/Notch* repeat region of *CNotch-1* and had the following sequence: 5′-CCAGCACTGCAGTGACTGTGAGC-3′. The 23-mer oligonucleotide was synthesized with phosphorothioate linkages between all bases. The oligonucleotide was extracted and precipitated to remove salts and organics. A missense oligonucleotide was used as a control. Cultures of the retinal cells, prepared as described above, were incubated in medium containing 25 μM oligonucleotide for 24 hours.

RESULTS

Mixed cell cultures

The main goal of this study was to examine environmental factors that influence ganglion cell determination in the developing retina. More specifically, the aim of this study was to determine whether differentiated cells in the developing retina release factors that influence the fate of cells that differentiate later. Young embryonic retinal cells at embryonic day 4 (E4), an age when ganglion cells are just starting to be generated, were cultured adjacent to other embryonic retinal cells taken from various ages, particularly ages at which ganglion cells had already developed. The two populations of cells were separated by a membrane that prevented cell-cell contact but that allowed soluble molecules to pass (Fig. 1), an approach used previously by Watanabe and Raff (1992). The thymidine analogue, bromodeoxyuridine (BrdU), was added to the culture medium to label dividing cells. This allowed the cells that divided in culture to be distinguished from those that differentiated in vivo. After 24 hours, the co-cultures were fixed and processed for immunohistochemistry using an antibody to BrdU and an antibody to RA4, which recognizes ganglion cells within minutes after mitosis (Fig. 2; McLoon and Barnes, 1989; Waid and McLoon, 1995). Cells that were double labeled with RA4 and BrdU were considered to be ganglion cells that developed in the culture system. This culture system was used to address the question of whether older retinal cells released a factor that prevents development of more ganglion cells.

More ganglion cells developed in the E4 retinal test population cultured adjacent to other E4 cells than when cultured adjacent to older populations of retinal cells (Fig. 3). When E4 cells were cultured adjacent to other E4 cells, 24% of the test population of cells that divided in culture, as evident by BrdU labeling, was also labeled with RA4. However, when E7

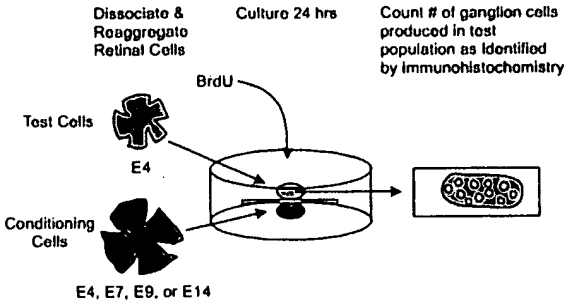


Fig. 1. Schematic of the mixed cell culture design. A test population of cells from E4 chick retina was dissociated, reaggregated and cultured opposite a similarly prepared population of cells from either E4, E7, E9 or E14 retina. The two populations were separated from each other by a membrane with pore size of 0.01 μm and cultured in medium containing BrdU. After 24 hours, the cultures were fixed and sectioned. Immunohistochemistry was used to quantify the number of ganglion cells produced in culture in the E4 test population.

retinal cells, an age at which more than half the ganglion cells have been produced, were cultured adjacent to E4 cells, 10% of the E4 test population that underwent division in culture was labeled by the RA4 antibody. When E4 cells were cultured adjacent to E9 or E14 cells only 5% of the test population that divided in culture were labeled with the RA4 antibody.

The effect of different aged conditioning populations on E4 test cells could have been due to a decrease in the rate of cell division in the younger population or to an increase in death of selected cells in the younger population. Cell division and cell death were quantified in the test cell population with each co-culture pairing. To assess differences in cell division, the

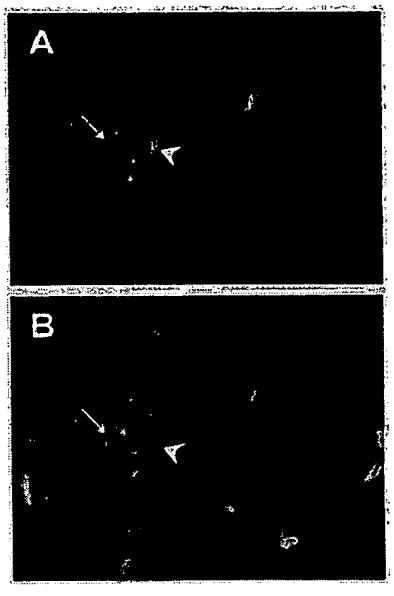


Fig. 2. Micrographs of a section from an E4/E4 co-culture stained (A) with an antibody to RA4 (red) to show ganglion cells and with DAPI (blue) to show all cells or (B) with an antibody to RA4 (red) and an antibody to BrdU (green) to show cells that divided in vitro. The arrow points to an RA4$^+$ cell with BrdU label in its nucleus, indicating a ganglion cell that underwent division in culture. The arrowhead points to a ganglion cell that did not undergo division in culture.

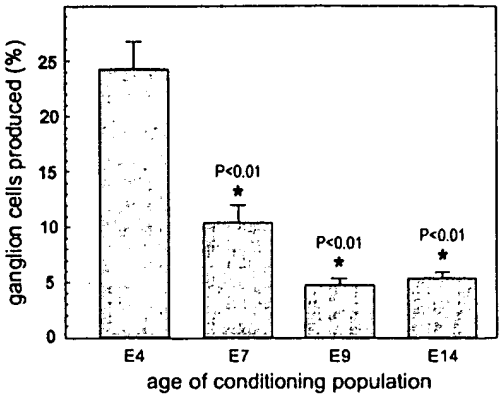


Fig. 3. Ganglion cell production was decreased in young retinal cell populations cultured adjacent to older retinal cells. The graph compares the percent of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the E4 test population when cultured adjacent to E4, E7, E9 or E14 retinal cells. Increasingly older conditioning cells, up to E9, were more effective at reducing ganglion cell production in the test cell population. Asterisks indicate ganglion cell production significantly different than in the E4/E4 co-cultures.

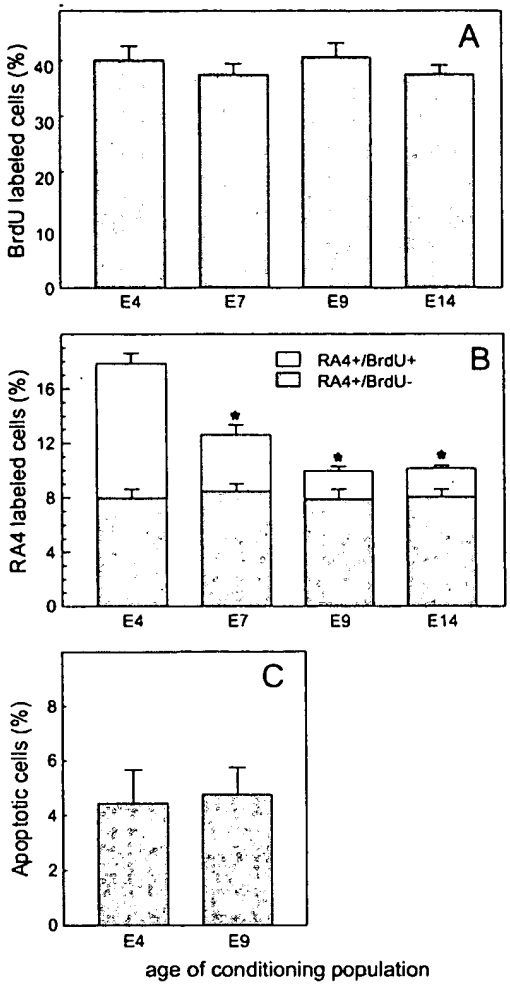


Fig. 4. Cell division and cell death in the test population were not affected by different-aged conditioning cells. (A) The graph compares the percentage of all cells in the E4 test population that divided in culture ($BrdU^+$). There were no statistically significant differences among the different experimental groups. (B) The graph compares the number of ganglion cells produced while in culture and produced in vivo in the test population for each conditioning population. The open bar represents ganglion cells that were produced in the culture ($RA4^+/BrdU^+$), and the solid bars represent the ganglion cells that were produced in vivo ($RA4^+/BrdU^-$). The differences among the different experimental groups in the number of ganglion cells were only in those produced in vitro; there were no statistically significant differences in the number of ganglion cells produced in vivo among the different experimental groups. This suggests that death of ganglion cells was not responsible for the differences in the number of ganglion cells. Ganglion cell death would be expected to affect ganglion cells produced in vivo and in vitro. (C) The graph compares the percentage of apoptotic cells in the E4 test population for each conditioning population. There were no statistically significant differences in the percentage of apoptotic cells among the different experimental groups.

percentage of cells that incorporated BrdU was determined in the test cell population for each culture condition. There was no statistical difference in the percentage of BrdU-labeled cells between any of the culture conditions examined (Fig. 4A). This excludes the possibility that the decrease in the percentage of $RA4^+$ cells observed, when E4 cells were cultured adjacent to older retinal cells, was due to a change in cell division. Cell death in the cultures was examined in two ways. First, if cell death targeted ganglion cells in certain conditions, then one would expect an overall reduction in the total number of ganglion cells in the test population including those produced in vitro and in those produced in vivo prior to removing the retinas to culture. The number of ganglion cells produced prior to culturing was quantified in the test population under each condition by counting the $RA4^+/BrdU^-$ cells. There was no significant difference in this population of cells between any of the culture conditions examined (Fig. 4B). The only significant differences in the number of ganglion cells among the different conditions were in those cells produced in vitro (i.e. $RA4^+/BrdU^+$, Fig. 4B). Second, the percentage of dying cells in the E4 test population was also compared among the various conditions. Dying cells were identified by end labeling DNA with a histochemical marker. Dying cells with fragmented DNA were heavily labeled. Again, no statistical difference was found in the percentage of dying cells between any of the culture conditions (Fig. 4C). Similarly, the number of pyknotic cells per square millimeter identified in Thionin-stained sections of the E4 test population from E4/E4 and E4/E9 cocultures were statistically the same. These data do not support the possibility that differences in cell death were responsible for the different number of $RA4^+$ cells observed in the different culture conditions. This suggests that older retinal cells secrete some factor that blocks development of ganglion cells.

A major change in retina from E4 to E7 to E9 is the progressive addition of more ganglion cells. It is possible that, once ganglion cells differentiate, they produce a signal that inhibits development of more ganglion cells. To examine this possibility, E4 retinal cells were cultured adjacent to an older population of cells that had very few ganglion cells. Retinas 'depleted' of ganglion cells were made by removing the tectum from embryos at a very young age and allowing the embryos to develop until E14. In the absence of the central target for their axons, the ganglion cells degenerate

prior to this age (Hughes and McLoon, 1979). Immunohistochemistry with the RA4 antibody confirmed the loss of retinal ganglion cells in these embryos by E14. When E4 cells were cultured opposite E14 retinal cells depleted of ganglion cells, 20% of the cells that divided in culture were RA4$^+$ (Fig. 5). However, when E4 cells were cultured opposite normal E14 retinal cells, only 5% of the cells that divided in culture were RA4$^+$. Both of these conditions showed similar rates of cell division and cell death in the test cell population. Thus, the results obtained with E14 retinal cells depleted of ganglion cells as the conditioning population looked similar to results obtained from E4/E4 co-cultures. In fact, there was no statistical difference between the number of ganglion cells produced in E4 cells when cultured opposite either other E4 cells or E14 cells depleted of ganglion cells (P=0.2). This result suggests that the ganglion cells in the older cultures are the source of a diffusable factor that blocks development of more ganglion cells.

Conditioned medium

The evidence points to a factor released from older retinal cells that inhibits ganglion cell development in this paradigm. It is possible that the older conditioning cells release enough factor that the culture medium would have sufficient activity to affect developing cells. If true, this would facilitate characterization of the factor. To test this hypothesis, culture medium was conditioned for 24 hours by cultures of dissociated E9 retinal cells. The medium was removed from the conditioning cultures, filtered and added to cultures of E4 retinal cells with BrdU to label dividing cells. After 24 hours, the cultures were fixed and assayed for ganglion cell production as described above. Fewer ganglion cells developed in the E4 retinal test population of cells cultured in E9 conditioned medium than when cultured in fresh medium. When E4 retinal cells were cultured in fresh medium, approximately 21% of the cells that divided in culture labeled with the RA4 antibody, compared to only 5% when cultured in conditioned medium (Fig. 6). It is possible that developing neuronal cells deplete conditioned medium of some needed nutrient for ganglion cell production. To test this, E6 forebrain cells were used to condition medium and E4 retinal cells were cultured in this conditioned medium. There was no significant difference in ganglion cell production between E4 cells cultured in fresh medium or forebrain-cell-conditioned medium (Fig. 6). Thus, E9 retinal cells apparently release a factor into medium with sufficient activity to alter cell fate. Furthermore, since E6 forebrain cells did not exhibit this activity, it suggests that the factor produced by retinal cells is not produced by all CNS neurons, or at least not at all stages of development.

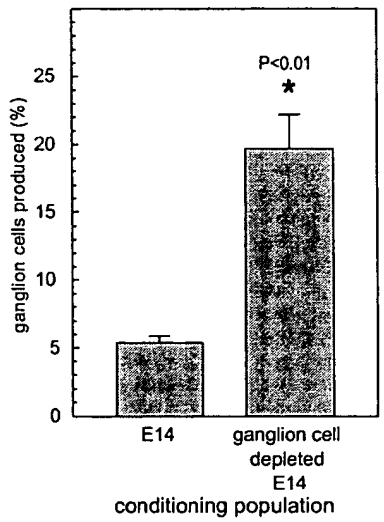


Fig. 5. Conditioning cell populations depleted of ganglion cells lost the ability to inhibit ganglion cell production in the E4 test cell population. The graph compares the percentage of cells produced in vitro (BrdU$^+$) that differentiated as ganglion cells (RA4$^+$) in the test cell population when cultured adjacent to E14 retinal cells or E14 retinal cells depleted of ganglion cells. Ganglion cell production in the test population was restored to that seen with E4 conditioning cells when older retina depleted of ganglion cells was used for the conditioning population. The asterisk indicates ganglion cell production significantly different than the E14 control.

In order to further characterize the factor, conditioned medium was heated to 70°C for 15 minutes, a treatment that denatures many proteins. E4 retinal cells were cultured in the heat-treated conditioned medium and, after 24 hours, were fixed and assayed for the production of ganglion cells. Approximately 6% of the cells that divided in culture were RA4$^+$, which is similar to the percentage of RA4$^+$ cells produced in untreated conditioned medium (Fig. 6). This indicates that the factor responsible for inhibiting ganglion cell production is heat stable.

In order to characterize the approximate size of the factor produced by older retinal cells that inhibits ganglion cell production, conditioned medium was filter fractionated into high (>10 kDa), medium (3-10 kDa) and low (<3 kDa) molecular mass components. Each conditioned medium fraction was combined with its missing fractions prepared with fresh medium. Each reconstituted medium was used to culture E4 retinal cells. After 24 hours, ganglion cell production was assessed in these cultures. The medium-sized fraction did not affect ganglion cell production significantly (Fig. 6). Whereas, E4 retinal cells cultured with the <3 kDa and >10 kDa fractions had a significant reduction in the percentage of ganglion cells that was produced in the culture compared to fresh medium. There was no significant difference between the number of ganglion cells produced when the E4 cells were cultured in the low molecular mass fraction or whole conditioned medium. This suggests that the major factor or factors produced by the older retinal cells, and possibly by ganglion cells, that inhibits ganglion cell production is <3 kD in size.

Notch

A previous study reported that activation of the Notch protein in progenitor cells inhibited ganglion cell production in developing chick retina (Austin et al., 1995). It is possible that the factor partially characterized in the present study activates Notch, or it may be part of a separate parallel pathway for controlling cell fate. If the latter is true, then one mechanism may dominate. To test the relationship between these factors, E4 retinal cells were cultured in medium conditioned by E9 retinal cells or in an antisense oligonucleotide to *CNotch-1*, as used in Austin et al. (1995) or in a combination of both. In fresh medium, the addition of antisense oligonucleotide to block *Notch* expression resulted in an increased number of ganglion cells produced in culture, as reported previously (Fig. 7). Conditioned medium, together with the antisense oligonucleotide, had no effect on the number of ganglion cells produced in the E4 population compared to E9 conditioned medium alone (Fig. 7). Cultures treated with

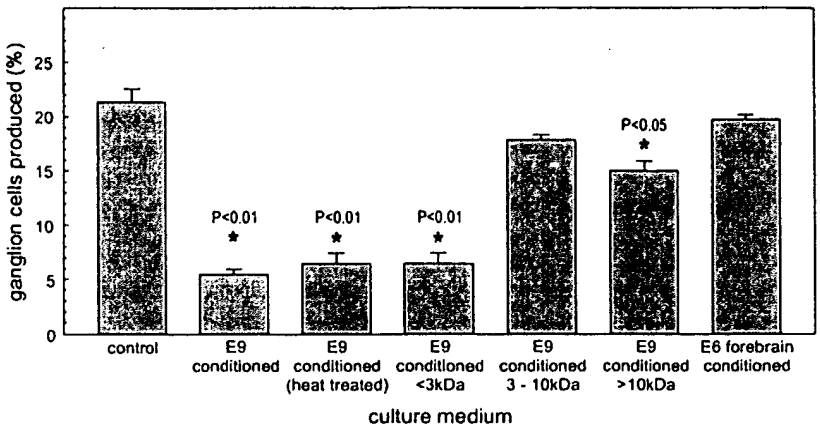


Fig. 6. The ganglion cell inhibitory factor recovered in medium conditioned by older retinal cells was <3 kDa and heat stable. Conditioned medium was harvested from 24 hour cultures of dissociated E9 retinal cells. The conditioned medium was used to culture reaggregated E4 retinal cells, the test cells. The graph compares the percentage of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the E4 test cell population in fresh medium (control), conditioned medium, conditioned medium heated to 70°C for 15 minutes, conditioned medium size fractionated and in medium conditioned by embryonic forebrain cells. Asterisks indicate ganglion cell production significantly different than the control.

missense oligonucleotides showed no change in ganglion cell production compared to cultures maintained in normal medium. This indicates that the factor produced by older retinal cells prevents cells from differentiating as ganglion cells even when *Notch* expression is reduced. This suggests that Notch may either play some other role than specifically directing the ganglion cell fate pathway or that its role is secondary and independent to the secreted factor produced by older retinal cells.

DISCUSSION

The primary aim of this study was to determine whether differentiated cells in the developing retina express factors that reduce further production of ganglion cells, one of the first cell types to develop in the retina. This was studied by culturing very young retinal cells, the test population, adjacent to retinal cells of various ages, the conditioning population, and then quantifying subsequent ganglion cell production in the test population. The presence of older retinal cells resulted in production of fewer ganglion cells in the test population. There was a greater effect with older conditioning cells up to embryonic day 9 (E9).

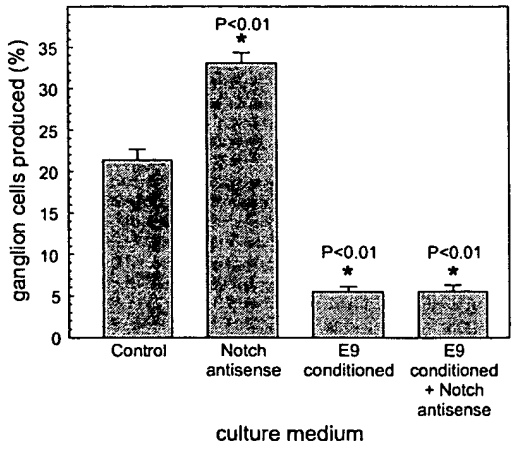


Fig. 7. Reducing Notch expression in cultures of E4 retinal cells with antisense oligonucleotides resulted in increased ganglion cell production, while medium conditioned by older retinal cells together with Notch antisense oligonucleotides resulted in reduced ganglion cell production. The graph compares the percentage of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the test cell population in fresh medium (control), medium with Notch antisense oligonucleotides added, medium conditioned by E9 retinal cells and in conditioned medium supplemented with antisense oligonucleotides. Asterisks indicate ganglion cell production significantly different than the control.

The effect of older retinal cells on the younger cell population was due to a secreted factor. The test cell population was separated from the conditioning cell population by a porous membrane. This prevented any direct cell-cell contact between the two populations but allowed soluble molecules to diffuse between the two populations. Medium conditioned by older retinal cells and then used to culture younger retinal cells also affected the number of ganglion cells that developed in the younger cell population. This also indicates that a factor secreted by older retinal cells blocked development of ganglion cells in the younger cell population. These findings are complementary to previous studies that showed older retinal cells secrete factors that promote production of rod cells in populations of younger retinal cells (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992). The approaches used in these previous studies were similar to those used in the present study. It is possible that the older retinal cells blocked expression of certain ganglion-cell-specific genes in the younger retinal cell population rather than reducing commitment of cells to the ganglion cell phenotype. These studies together, however, suggest that older retinal cells secrete factors that cause progenitors in the younger population to produce rods and possibly other cell types instead of producing ganglion cells.

The factor produced by older retinal cells that altered ganglion cell production most likely acted only on cells undergoing division rather than switching the fate of cells that were postmitotic. The presence of the conditioning population resulted in changes in the number of ganglion cells in the test population that divided in culture. BrdU labeling was used to identify the cells that divided in culture. There was a change with different aged conditioning cells only in the number of ganglion cells labeled with BrdU; the number of ganglion cells not labeled with BrdU in the test population was statistically the same for all experimental conditions. Previous studies linked environmentally induced changes in cell phenotype to cell division. Cell cycle progression has been shown to be coupled to expression of *even-skipped*, a gene required for neuronal specification in *Drosophila* (Weigmann and Lehner, 1995; Cui and Doe, 1995). Blocking the

cell cycle also prevented the expression of *even-skipped* and neuronal development, suggesting that neuronal differentiation is linked to the cell cycle. Work on vertebrates also suggests that cell division is required for cells to acquire a phenotype appropriate for their environment (McConnell and Kaznowski, 1991). Half of the cells transplanted from embryonic ferret cortex to postnatal cortex migrated to laminar positions appropriate for cells born in postnatal embryos while the remainder migrated to positions appropriate for cells born in embryonic cortex. Those cells that migrated to positions appropriate for their new environment underwent at least one round of cell division after transplantation. Thus, it may be a general phenomenon in the central nervous system that induction of specific cell phenotypes is intimately linked to the cell cycle.

Differentiated cells in the conditioning cell population in the present study most likely secreted the factor that altered cell determination of younger retinal cells. The main difference in the retina with increasing age is the addition of more differentiated cells. Furthermore, it is likely that differentiated ganglion cells specifically are responsible for secreting the factor. The majority of ganglion cells in the chick retina are produced between the ages of E3 and E9 (Fujita and Hori, 1963; Kahn, 1974; Spence and Robson, 1989; Snow and Robson, 1994; Waid and McLoon, 1995). The progressive addition of ganglion cells with increasing age paralleled the effectiveness of different aged conditioning cells in reducing ganglion cell production in the test population. The effectiveness of older conditioning cells in reducing ganglion cell production plateaued at E9, the same age at which ganglion cells reach their peak number. Tectal ablations were used to generate older conditioning cell populations depleted of ganglion cells (Hughes and McLoon, 1979). Older conditioning cell populations depleted of ganglion cells did not alter the number of ganglion cells that developed in the young test population. This suggests that as ganglion cells develop in the normal retina, they release a factor that blocks development of more ganglion cells.

The results of another study suggested that differentiated amacrine cells might also inhibit further development of their own cell type (Reh and Tully, 1986). When dopaminergic amacrine cells were depleted in developing retina, the next cells to differentiate included an abnormally high percentage of dopaminergic amacrine cells. When these animals were allowed to survive longer, the period of increased production of dopaminergic amacrine cells was followed by a period of reduced production of this cell type. An alteration in the number of dopaminergic amacrine cells was not accompanied by a change in the number of other amacrine cell types. Thus, it was suggested that cells were recruited to the dopaminergic amacrine cell type from an uncommitted pool of cells rather than causing a switch in the transmitter type within a pool of amacrine cells. The interpretation of these findings is that dopaminergic amacrine cells produce a factor that acts locally to reduce further production of the same cell type. It may be a general mechanism in nervous system development that differentiated cells inhibit further production of more of their same cell type.

The identity of the factor secreted by older retinal cells that blocks development of more ganglion cells is not yet known. The factor, as present in medium conditioned by older retinal cells, was partially characterized. The factor was heat-stable, and the majority of the activity remained in a <3 kDa fraction. A previous study with rat suggested that the amino acid, taurine, was responsible for promoting rod cell production in medium conditioned by older retinal cells (Altshuler et al., 1993). Taurine is heat-stable and <3 kDa. Taurine is expressed transiently in high levels by ganglion cells shortly after they differentiate in rat retina (Lake, 1994). It is possible that taurine is responsible for switching cells from the ganglion cell fate in the present study as well. Another study, however, failed to show a similar effect of taurine on chick retinal cells, even though it showed that taurine increased the number of rods that developed in rat retina (Kirsch et al., 1996). In chick, taurine may block development of ganglion cells while increasing production of cell types other than rods. A small but significant reduction in ganglion cell production was also observed with the >10 kDa fraction of conditioned medium. A previous study observed that a >10 kDa fraction of medium conditioned by older retinal cells inhibited rod cell production (Altshuler et al., 1993). This activity could be related to the high molecular mass activity observed in the present study. Further work is needed to identify the factors secreted by older retinal cells in developing retina.

The finding that older retinal cells secrete a factor that blocks development of ganglion cells is somewhat at odds with recent findings, which indicate that the Notch protein has a similar function in chick retina (Austin et al., 1995; Ahmad et al., 1997; Henrique et al., 1997). *Notch-1* is expressed in the proliferative zone of the developing retina. When Notch activity was increased by transfection of a constitutively active form of *Notch-1* or with a Notch ligand, Delta, there was a reduction in ganglion cell production. Conversely, blocking *Notch-1* or *Delta-1* expression with antisense oligonucleotides resulted in increased ganglion cell production. One interpretation of these results is that Notch specifically regulates ganglion cell production. The Notch protein is a transmembrane receptor that is activated by the cell surface ligands, Delta and Serrate/Jagged (Arvantis-Tsakonas et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996). Thus, Notch is believed to function via cell-cell contact. In the present study, the ability of older retinal cells to regulate ganglion cell genesis appears to be mediated by a secreted factor. It could be that this secreted factor represents a novel Notch ligand or a completely independent regulatory mechanism. To test this, young retinal cells were cultured with both *CNotch-1* antisense oligonucleotides and medium conditioned by older retinal cells. While the antisense oligonucleotide alone increased ganglion cell production, in combination with medium conditioned by older retinal cell, there was a reduction in ganglion cell production. This suggests that the secreted factor and Notch regulate ganglion cell production through separate pathways.

Another possibility is that Notch plays a more general role in retinal cell differentiation. Activation of Notch may prevent differentiation of any cell type and differentiation into specific cell types may be controlled by other factors (Bao and Cepko, 1997; Henrique et al., 1997). In *Drosophila*, activation of the Notch protein in progenitor cells appears to prevent the cells from committing to any particular fate (Fortini et al., 1993). R8 photoreceptors are normally the first cell type to differentiate in the *Drosophila* eye. Blocking Notch expression in the *Drosophila* eye caused most of the cells posterior to the morphogenetic furrow to differentiate immediately as R8 cells (Cagan and Ready, 1989). Similarly, overexpression of Notch or the Notch ligand, Delta, in developing *Xenopus* retina resulted in an increase in the number of progenitor cells and a

decrease in all types of differentiated cells (Dorsky et al., 1995, 1997). Thus, factors secreted by differentiated ganglion cells may promote differentiation of other retinal cell types, but only in cells in which Notch is not active.

In summary, this study suggests that as cells differentiate in the developing retina, they secrete factors that prevent development of ganglion cells and may possibly promote development of other cell types. Ganglion cells appear to be the likely source of the factors. The ganglion cell phenotype appears to be the default pathway for differentiation in the developing retina. In the absence of any environmental signal, retinal cells will differentiate as ganglion cells. It is likely that the secreted factor produced by ganglion cells prevents all cells in the developing retina from differentiating into ganglion cells. A similar function has been attributed to cell-contact-mediated signaling through Notch and Delta. It appears that secreted factors and Notch signaling represent separate control mechanisms. It may be that the secreted factors directly influence cell fate decisions, while the Notch pathway controls a more general decision of whether or not to differentiate.

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Regulation of neural stem cell differentiation in the forebrain

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Summary In the developing forebrain, mounting evidence suggests that neural stem cell proliferation and differentiation is regulated by growth factors. *In vitro* in the presence of serum, stem cell proliferation is predominantly mediated by fibroblast growth factor-2 (FGF-2) whereas neuronal differentiation can be triggered by FGF-1 in association with a specific heparan sulphate proteoglycan. On the other hand, astrocyte differentiation *in vivo* and *in vitro* appears to be dependent on signalling through the leukaemia inhibitory factor receptor (LIFR). The evidence suggests that in the absence of LIFR signalling, the stem cell population is present at approximately the same frequency and can generate neurons but is blocked from producing astrocytes that express glial fibrillary acidic protein (GFAP) or have trophic functions. The block can be overcome by other growth factors such as BMP-2/4 or interferon-γ, providing further evidence that the inhibition to astrocyte development does not result from loss of a precursor population. Signalling through the LIFR, in addition to stimulating astrocyte differentiation, may also inhibit neuronal differentiation, which may explain why this receptor is expressed at the earliest stages of neurogenesis. Another signalling system which also exerts its influence on neurogenesis through active inhibition is Delta-Notch. We show *in vitro* that at high cell densities which impede neuronal production by FGF-1, lowering the levels of expression of the receptor Notch by antisense oligonucleotide results in a significant increase in neuronal production. Thus, stem cell differentiation appears to be dependent on the outcome of interactions between a number of signalling pathways, some which promote specific lineages and some which inhibit.

Key words: cortical development, glia differentiation, growth factors, leukaemia inhibitory factor, neuron differentiation.

Introduction

Concept of the neural stem cell

The concept of a stem cell whose properties include the ability to give rise to a multitude of cell types and to self-renew has been well established in many systems, particularly the haematopoietic system, and yet it is only recently been deemed applicable to the central nervous system. This was partly because we were unable to grow stem cells *in vitro* or to monitor their progeny *in vivo*. Perhaps a more important impediment to the acceptance of the stem cell concept has been the unwillingness to embrace the property of self-renewal, mainly because this implied an ongoing presence of stem cells in the mature nervous system.

The last few years has, however, provided cogent support for this concept through the ability to grow populations of stem cells *in vitro*[1,2] and then, more importantly, to clone individual stem cells and formally demonstrate their multipotentiality and self-renewal properties;[3,4] the use of retroviral markers to demonstrate the multipotential character of stem cells *in vivo*;[5] and finally, the identification of stem cells in the brains of animals at times beyond the neurogenic period[6] and into adulthood,[7,8] confirming the considerable extent of self-renewal within the stem cell population.

It is not the intention of the present review to give an overview of stem cell biology because we have done this elsewhere;[9] instead we will focus on results, predominantly from our own laboratories, which address the mechanisms regulating the differentiation of stem cells in the forebrain of embryonic and adult mice.

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Fibroblast growth factor and stem cell regulation

Proliferation

The first suggestion that the fibroblast growth factor (FGF) family may influence stem cell growth came from *in vitro* studies which demonstrated that of a variety of growth factors tried, FGF-2 and FGF-1, in the presence of serum and insulin-like growth factor (IGF)-1, were the most effective agents in stimulating cell division in populations of neuro-epithelial cells obtained from the embryonic day-10 (E10) mouse forebrain.[1,2] Although it was demonstrated that both neurons and astrocytes could be generated from dividing cells, no conclusion could be drawn about the

nature of the precursor because the experiments were performed at high cell density.

The subsequent demonstration that FGF-2 could be used to stimulate single cells from E10 neuro-epithelium to produce clones consisting of several thousand cells which, in the presence of a glial-derived conditioned medium, produced neurons in addition to astrocytes, confirmed the suspicion that FGF-2 could stimulate the proliferation of stem cell populations.[4] The frequency of precursors with the ability to give rise to clones of a significant size (> 100 cells) was on average ~5% of the population. Close examination of the proliferation curves obtained from bulk cultures stimulated with FGF-2[1] also suggested that the majority of cells generated at the end of a 3-day culture period arose from a small subpopulation of cells no larger than 10%. A similar frequency of cortical clones was observed under culture conditions that did not include serum.[10] Thus, approximately one in 10 cells in the E10 cortical neuro-epithelium has the ability to generate a large number of progeny even though it is clear that > 99% of cells at this stage are dividing.[2] The remaining dividing cells appear to undergo fewer divisions and generate a small number of progeny, as is evident using retroviral markers to identify cortical clones *in vivo*. Alternatively, they do not respond to FGF-2. Clearly, there is a hierarchy in both the proliferative and lineage potential of precursor cells within the developing forebrain, but a hallmark of the true stem cell is their ability to generate large numbers of progeny. In addition, the FGF-2 responsive clones have the property of self-renewal: > 80% of the clonal progeny cells gave rise to new clones.[4]

Differentiation

Although our initial studies of high-density cultures indicated that FGF-2 could generate neurons, especially at higher concentrations, subsequent clonal examination revealed that the FGF-2-stimulated clones in the presence of serum rarely produced neurons, although astrocytes did arise.[4] Subsequent studies revealed that neuronal differentiation could be inhibited by FGF-2, which appears to predominantly drive proliferation. Earlier, however, it was observed that immortalized precursors from the E10 forebrain did differentiate in response to FGF-2,[3] and subsequent studies in serum-free medium[10] have shown that cortical clones generated in high doses of FGF-2 contain both neurons and oligodendrocytes. It was also reported that clones generated in low levels of FGF (0.1 ng/mL) contained only neurons, as did the small number of clones arising without FGF-2.[10] This suggests several things: first, that serum inhibits neuronal generation and second, the level of FGF-2 determines the cell-type constituents of the clone. However, closer examination of these results reveals their similarity to the serum-generated clones because the number of neurons generated per clone, regardless of FGF-2 concentration and clone size, is small (average of 15). Thus, like the serum-derived clones, FGF-2 at higher concentrations, which generate substantial-sized clones, appears to inhibit further neuronal production by the stem cell. Whether low doses of FGF-2 actually induce neuronal differentiation seems, as the authors state, [10]unlikely, but it is compatible with a primary role in expanding the precursor population. In addition, like the serum-plus FGF-2 clones, the large serum-free clones contain a majority of glial cells. There is, however, one major difference: the serum-plus clones contain astrocytes with virtually no oligodendrocytes whereas the serum-free clones are almost exclusively comprised of oligodendrocytes. Astrocyte production requires additional factors provided by glial conditioned medium, which as we will discuss, may function by stimulation through the leukaemia inhibitory factor (LIF) receptor complex. It is well established that oligodendrocyte production is enhanced by serum-free conditions.[11]

Thus, it appears that the production of neurons from the majority of precursors within a large clone requires additional factors to FGF-2; Ghosh and Greenberg showed that neurotrophin-3 (NT-3) could stimulate neurogenesis in FGF-2-stimulated cultures,[12] and we have shown that conditioned medium from an astrocyte cell line can result in a significant number of FGF-2 expanded clones producing neurons after FGF-2 withdrawal.[4,13] One strong candidate for providing a neurogenic stimulus was FGF-1 since we had demonstrated that it was expressed at E11 just as neurogenesis begins in the mouse cortex; whereas FGF-2 was present much earlier at E9.5 prior to the commencement of neuronal production. However, initially we were not able to demonstrate a differential action of FGF-1 compared to FGF-2 in our cultures regardless of the presence or absence of heparin. Nevertheless, Guillemot and Cepko had shown that FGF-1 was far more potent than FGF-2 in promoting the differentiation of retinal ganglion cells.[14]

Role of heparan sulphate proteoglycans in FGF responsiveness

In the process of demonstrating that FGF-1 and FGF-2 were produced by neuro-epithelial cells from mouse forebrain, it was discovered that the majority of the FGF was bound to a single dominant heparan sulphate proteoglycan (HSPG)[15,16] which we have since identified as a variant of Perlecan.[17] The most interesting finding, however, was the binding specificity of this HSPG isolated from the developing forebrain at different times: HSPG from E10 brains (HS-2) predominantly bound FGF-2, whereas HSPG from E12 brains (HS-1) preferentially bound FGF-1.[15] Recently we have shown that this shift is associated with an increase in the number of sulphated domains and increased heparan sulphate glycosaminoglycan (HS) side-chain length.[18] It is known that the charge-domains created by sulphation are critical to FGF-1 and FGF-2 binding and also are thought to influence interaction of FGF with its cognate receptor(s). One current hypothesis which we favour is that HS serves to couple FGF to specific HS-binding regions on specific FGF receptors (FGFR) to form an activated signalling complex of FGF/HS/FGFR.

It was found that precursor proliferation in high-density cultures was significantly enhanced when FGF-1 was used with HS-1, or FGF-2 with HS-2,[15] confirming the importance of this type of presentation mechanism. It

provides a mechanism by which cell activation can be regulated without the requirement for stringent regulation of FGF concentration or receptor number, and is probably used by a number of the heparin-binding growth factors.

Recently we have used FGF-1 in combination with HS-1 and shown that > 80% of the clones generated contain large numbers of neurons (> 100), whereas less than 3% of clones have neurons when heparin is used (PF Bartlett, V Grefarath and M Ford, unpubl. obs. 1998).

The precise mechanism by which neuronal signalling occurs is not known, but it probably involves the differential signalling through one or more of the FGFR on the stem cell's surface. We and others have shown that isoforms of FGFR 1, 2 and 3 are expressed on the precursor population in developing cortex during this period[10,14] so the question remains as to which receptor signals neurogenesis and which signals proliferation.

Stem cells from the adult forebrain

So far we have mentioned only the responsiveness of embryonic stem cells; however, it became clear that there was a population of precursors within the adult forebrain which could be stimulated to produce neurons in response to FGF.[7] More recent clonal experiments using precursors from the SVZ of the lateral ventricle of adult mice have shown that the adult stem cell responds in a similar way to its embryonic counterpart, forming large undifferentiated clones in response FGF-2 at the frequency of ~1 in every 200 cells plated. Again, recent experiments have shown that FGF-1 can stimulate neuronal production in these clones, but does not require the addition of HS-1 to these cultures (GJ Brooker and PF Bartlett, unpubl. obs. 1998). This suggests there is either a different array of receptors, or there is endogenous HSPG on the cell membrane which can present FGF-1 in the appropriate manner.

The only stem cell population we have found that produces neurons in clonal cultures in response to FGF-2 is contained within the E17 forebrain population.[6] The explanation for this is not clear, but it may indicate that this precursor population isolated just after the termination of neurogenesis has received the appropriate signals prior to isolation, whereas the precursor from the adult has resumed a more embryonic state.

Factor regulation of astrocyte differentiation

As discussed in the previous section, there is good evidence for the bi-potential stem cell's choice of lineage being determined, at least in part, by environmental factors such as growth factors. Previously we had shown, *in vitro*, that leukaemia inhibitory factor (LIF) could stimulate precursors from the E10 spinal cord to express glial fibrillary acidic protein (GFAP).[19] In addition, the present study also showed that antibodies to the LIF receptor (LIFR) significantly reduced the number of astrocytes that developed in the absence of exogenous growth factors, suggesting that endogenous ligands acting through the LIFR influence astrocyte development. Other ligands that signal through the LIFR complex (a heterodimer composed of LIFR and gp130) such as ciliary neurotrophic factor (CNTF), also have been shown to promote GFAP expression in central nervous system (CNS) precursor populations.[20] Thus, the *in vitro* results strongly suggest that ligands that signal through the LIFR complex may have a role in regulating astrocyte differentiation.

The role of LIFR in regulating astrocyte production was supported by the demonstration that E19 embryonic mice with a targeted disruption of the low-affinity LIF receptor gene, which appear to have normal CNS development, have a deficiency of GFAP-positive cells in the developing hindbrain.[21] Unfortunately, because these animals die at E19 (which is just 2 days after the first appearance of GFAP[22]) it was difficult to determine whether this astrocyte deficiency was due to general retardation in development or a failure in astrocyte generation due to lack of signalling through the LIF receptor. To explore these possibilities further, the properties of precursor cells from the forebrain of LIFR-deficient mice were examined *in vitro*.[23] It was shown that precursors from the forebrains of mice homozygous for the LIFR null mutation (LIFR–/–) failed to generate significant numbers of GFAP-positive cells even after 3 weeks *in vitro*. To determine if the lack of GFAP expression in LIFR–/– precursors fully reflected a failure in astrocyte development, an assay to assess astrocyte function was performed. Previously it has been shown that astrocytes promote neuronal differentiation and/or survival in a number of systems;[4,24] thus, the ability of established monolayers derived from LIFR +/+, +/–, and –/– forebrains to support the neuronal differentiation was tested. No difference was found in the number of neurons produced on the LIFR +/+ or +/– monolayers, but there was ~10-fold fewer neurons found on the LIF–/– monolayers.[23] The study showed that signalling through the LIFR is required for the generation of functional astrocytes, not just for the expression of GFAP. This is an important point because it has recently been shown that one of the downstream signalling pathways activated by signalling through LIFR, the JAK-STAT pathway, can directly activate the GFAP gene. It has been shown that STAT 3 can directly bind to a consensus site in the promoter region of the GFAP gene.[25] Thus, the regulation of GFAP expression can be directly regulated through the LIFR complex: both LIFR and gp130 appear to be required for this signal.[25]

It was subsequently shown that the precursor population in the LIFR–/– forebrain was in fact present because stimulation with bone morphogenetic protein (BMP)-2, a member of the transforming growth factor-β (TGF-β) growth factor family previously shown to stimulate GFAP expression in astrocytes, contained a significant percentage of GFAP-positive cells after 10 days *in vitro*. In addition, long-term passaging *in vitro* (> 5 weeks) revealed significant numbers of GFAP cells in LIFR–/– cultures which supported neuron generation and/or survival.[23]

We also found that there was no decrease in the total number of neural clones generated from the LIFR–/– mouse forebrain precursors with FGF-2; also strongly suggesting that LIFR signalling was not essential for the maintenance of precursor cells. As mentioned in the previous section, we had shown that FGF-2-stimulated forebrain precursors

have the ability to generate two types of clones: clones that contain both neurons and glia, or clones restricted to astrocytes. However, because the frequency of neuron-containing clones generated with FGF-1 and HSPG-1 is also unaltered in the LIFR-/- population, it suggests that there is no change in the relative frequency of either the bipotential or astrocyte-restricted clones in these animals.

The question arises as to whether signalling through the LIF receptor instructs a precursor to become committed to the astrocyte pathway. Several pieces of evidence support such an hypothesis: first, it has been shown that in the presence of LIF > 80% of precursors become GFAP positive *in vitro*;[19] second, that STAT-3, which is directly activated by LIFR signalling, can bind to the promoter region of the GFAP gene and regulate its expression;[25] and third, that stimulation with LIF can significantly inhibit the neuronal differentiation of clones (GJ Brooker and PF Bartlett, unpubl. obs. 1998). This latter finding is also true in clones derived from adult subventricular zone (SVZ). Although this favours the idea that signalling through the LIFR may actively promote astrocyte differentiation, an alternative interpretation is that LIFR signalling may inhibit neuronal differentiation leading to astrocyte production by default. Thus, it may be that LIFR signalling actively keeps the precursor in an undifferentiated, or stem cell state: as it does for pluripotential embryonic stem cells. Thus, neurogenesis may result from individual stem cells overcoming this inhibitory signal. A candidate for mediating this type of action is the recently discovered suppressors of cytokine signalling (SOCS) family, which have been shown to inhibit signalling through the LIFR.[26]

It is not known which LIFR ligand mediates this effect; we have shown that mice with a targeted deletion in the LIF gene have reduction in the number of astrocytes in the hippocampus but it is in no way complete.[23] Because CNTF also has been shown to promote astrocyte formation, it also may play a part. Also, other ligand–receptor pathways may replace LIFR at later stages of development. The finding that long-term cultures from LIFR mice do ultimately start to express GFAP and are functionally active supports this idea, as do recent experiments in which portions of LIFR-/- brains were transplanted to a syngeneic recipient and shown to contain GFAP cells several weeks after transplantation (PF Bartlett and AR Harvey, unpubl. obs. 1998).

Neuronal differentiation by DisInhibition

The concept raised in the previous section whereby neurogenesis results from overcoming signals that favour the maintenance of a stem cell state is best exemplified by the action of the neurogenic genes *Delta* and *Notch*, which code for a cell surface ligand and receptor, respectively, through a process of lateral inhibition which prevents adjacent precursors from differentiating. This process has been well demonstrated to regulate neurogensis in *Drosophila* and *Xenopus*, and more recently it has been shown to play a role in mammalian retinal differentiation.[27]

The key step in this phenomenon is the ability of a single precursor to express more of the ligand Delta than its neighbours, thereby activating the neighbour's Notch receptor signalling pathway, which inhibits neurogenesis by inhibiting the production of the helix–loop–helix transcriptional regulators Neurogenein and Neuro-D; which in turn regulate Delta levels. To investigate whether the action of the growth factors FGF-2 and FGF-1 could influence this pathway, we have begun to examine neuronal production in high cell density conditions where, as we have previously shown,[1] FGF-1 and FGF-2 promote proliferation rather than neuronal differentiation. When Notch-1 expression is reduced by the addition of antisense oligonucleotides to the cultures, it was found that in the presence of FGF-1, but not FGF-2, there was a significant increase in the number of neurons generated (CH Faux, A Turnley and PF Bartlett, unpubl. obs. 1998). Again, this demonstrates the predilection of FGF-1 to promote neuronal differentiation compared to FGF-2 (at similar concentration). It also suggests that neurogenesis *in vivo* may require both inhibition of Notch signalling and activation of FGF receptor signalling, although there may be a common mechanism whereby Notch expression is further reduced by FGF-1 signalling to levels below the threshold for inhibition. All these possibilities are presently being explored.

Inhibitory mechanisms in stem cells in the adult SVZ

We have also recently obtained evidence for a similar inhibitory mechanism acting on the precursor population in the adult SVZ.

As reported by Lois and Alvarez-Bullya,[28] explants of SVZ grown *in vitro* do not generate neurons from dividing cells. However, we have recently shown that the dividing cells within the explant have the propensity to give rise to neurons when dissociated and replated at clonal or low cell density. Replating at high cell density leads to inhibition of neurogenesis. The results suggest an inhibitory mechanism similar to lateral inhibition and both Delta and Notch are expressed in the adult SVZ (CH Faux and PF Bartlett, unpubl. obs. 1998). Such inhibitory mechanisms may restrict the ability of precursors within the SVZ to generate neurons apart from those destined for the olfactory bulb. It could be postulated that the olfactory stream provides signals that may reduce these inhibitory effects.

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